

lib

**Studies of the Molecular Basis of
Epidermolysis Bullosa Simplex**

Frances J. D. Smith

**A thesis presented for the degree
of Doctor of Philosophy
University of Edinburgh, 1994**



Abstract

The term epidermolysis bullosa (EB) encompasses a range of inherited bullous disorders, in which blisters develop on the skin and sometimes the mucous membranes in response to relatively mild mechanical trauma. The disease is subdivided into three main types; simplex, junctional and dystrophic, on the basis of clinical appearance, pathogenesis and the ultrastructural level of separation within the skin. The simplex form (EBS) is characterised by intraepidermal separation of the skin, due to cytolysis in the subnuclear region of the basal keratinocytes.

The aim of this project was to investigate the possible mechanisms for this intraepidermal blistering: excessive proteolytic activity within or around the basal cells, or the presence of a mutant temperature-sensitive structural protein within the basal cells. However, early in the project, discoveries by several research groups indicated that single point mutations in either of the basal cell keratins, K5 or K14, were probably primarily responsible for the increased cell fragility of the keratinocytes in at least some cases of EBS. An enzyme abnormality was therefore unlikely to be directly involved in the pathogenesis of the disease, although it could be a secondary factor. Attention was therefore concentrated on locating mutations in the genes encoding keratins K5 and K14, the putative defective genes, in five families affected by the Weber-Cockayne form of EBS, characterised by blisters predominantly on the hands and feet. DNA sequences of keratins K5 and K14 obtained from cDNA extracted from cultured keratinocytes, or from genomic DNA extracted from whole blood, were supported by clinical features and genetic linkage analysis using probes within or close to the keratin gene clusters on chromosomes 12 (type II keratins) and 17 (type I keratins). Keratin proteins expressed by the cultured keratinocytes were also examined by SDS polyacrylamide gel electrophoresis and immunoblotting.

In four of the five families examined, single nucleotide substitutions resulting in an amino acid change were identified. In two families, mutations were identified in the central non-helical L12 linker domain, one in keratin 5 and the other in keratin 14. This was surprising as relatively little is known about this region, and it was not considered to be particularly important in keratin filament assembly or network formation. Another mutation has been reported in this region in keratin 14 in a family with EBS-Koebner characterised by moderate and generalised blistering. Although just two residues away from the mutation reported here, the difference in severity of the resulting phenotypes emphasises the contribution of individual residues to the overall functioning of the keratin filament network. In the third family a mutation was identified in the 1A domain of keratin 5, at the carboxy terminal of this domain. In the fourth family a tentative mutation was identified near the centre of the 2B domain of keratin 14. Extensive sequencing of both K5 and K14 DNA from members of the fifth family did not identify any sequencing discrepancies and genetic linkage analysis suggested that the disease phenotype in this family was linked to the type I keratins. It is possible though, that mutations in minor basal cell keratins such as keratin 15, or in a keratin associated protein, could lead to the same clinical phenotype.

Keratin mutations have now been identified in another bullous disorder, bullous congenital ichthyosiform erythroderma, in the supra basal keratins K1 and K10. Taken together, these findings indicate that these pathological mutations are clustering to certain regions of the keratin molecule. The position of the mutation within the keratin molecule, rather than in which member of the heterodimer the mutation occurs, appears to determine the severity of the phenotype. The more severe phenotypes are due to mutations in the highly conserved terminal ends of the α -helical rod domain, and the milder phenotypes are due to mutations internal, or just outside these terminal ends of the rod domain. Not only has the cause of at least some cases of EBS now been identified but, collectively, these studies provide evidence for a structural function of

the keratin intermediate filament network within the cell. As yet there are no effective therapies to treat the clinical symptoms of EBS, but gene therapy may become possible to correct the genetic defect.

I declare that the studies presented in this thesis are the result of my own investigations.

Dr. M. J. Tidman and Dr. H. Horn recruited, and obtained clinical material from patients for the study. Keratinocytes were also cultured from the same patients in Professor I. M. Leigh's laboratory (Experimental Dermatology Department, Royal London Hospital) and some of this material was used in addition to my cultured keratinocytes. This work has not been submitted for any other degree.

Frances Smith

I dedicate this thesis to my parents Alison and Brian Smith

Acknowledgements

I would like to thank DEBRA for the award of my research studentship. I thank my supervisors, Dr. G. C. Priestley, Dr. M. J. Tidman and Professor E.B. Lane for all their encouragement and guidance in directing my project. To Dr. H. Horn, I am very grateful for her help in contacting and tracing EBS families and to members of these families for participating in the study. I thank members of the Medical Genetics Laboratory, Ninewells Hospital, Dundee for the control DNA samples and the Western General and the Royal Hospital for Sick Children, Edinburgh, for control skin samples. To members of Professor Lane's laboratory, especially Liz and Irwin, thank you all for your help, advice and friendliness.

A special thank you to all my friends especially Martina, Marina and Bruce, to my parents, grandmother and Jenny for all their help and support.

Table of Contents

Abstract

Acknowledgements

Abbreviations

Chapter 1. General Introduction	1
1.1 Epidermolysis Bullosa (EB)	2
1.2 The Human Skin	2
1.3 Classification of EB	6
1.4 Prevalence of EB	9
1.5 EB-like Disorders in Animals	10
1.6 Epidermolysis Bullosa Simplex (EBS)	11
1.7 Treatment of EB Simplex	22
1.8 Pathogenesis of EB Simplex	24
1.9 Theories on the Cause of EB Simplex	
1.9.1 A Proteolytic Enzyme Abnormality	24
1.9.2 Structural Abnormalities of the Keratinocyte Cell Membrane	28
1.9.3 A Defective Structural Protein in the Basal Keratinocytes	29
1.10 Intermediate Filaments	32
1.11 Keratins	34
1.12 Structure of Intermediate Filaments	37
1.13 Evolution of Intermediate Filaments	41
1.14 Intermediate Filament Assembly	42
1.15 Dynamic Properties of Intermediate Filaments	45
1.16 Deletion and Point Mutagenesis Studies of Keratins	46
1.17 Aim of Project	51

Chapter 2. Enzyme Studies	52
2.1 Effect of Neutral Blister Fluid on Skin Explants	53
2.2 Protease Activity in Cultured Keratinocytes and Whole Skin	54
 Chapter 3. Keratin Studies: Methods	 57
Cell Culture	
3.1 Primary Keratinocyte Cell Culture	59
3.2 3T3 Feeder Cells	61
3.3 Mycoplasma Testing of Cultured Cells	62
3.4 Immunocytochemical Staining of Keratinocytes	63
 DNA Analysis	
3.5 Extraction of mRNA from Cultured Keratinocytes	64
3.6 Reverse Transcription of mRNA to cDNA	65
3.7 Isolation of Genomic DNA from Whole Blood	66
3.8 Polymerase Chain Reaction (PCR)	67
3.9 Purification of Oligonucleotide Primers	68
3.10 Purification of PCR Amplified DNA	69
3.11 Double Stranded DNA Cycle Sequencing	71
3.12 Exclusion of Polymorphisms	72
3.13 Genetic Linkage Analysis	75
 Protein Analysis	
3.14 Extraction of Cytokeratins From Keratinocyte Cultures	79
3.15 SDS Polyacrylamide Gel Electrophoresis	79
3.16 Western Blotting	81
3.17 Staining of Western Blots with Anti-keratin Antibodies	81
3.18 Staining of Western Blots for Total Protein	82

Chapter 4. Keratin Studies: Results	84
4.1 Keratinocyte Cell Culture	85
4.2 Families A and B: Keratin Gene Mutations in the L12 Linker Domain	89
4.3 Family C: A Point Mutation in the 1A Domain of Keratin 5	122
4.4 Family D: A Point Mutation in the 2B Domain of Keratin 14	136
4.5 Family E: No Mutation Identified in Keratin 5 or Keratin 14	145
 Chapter 5. Discussion	 158
5.1 Different Approaches that Led to the Same Conclusion	159
5.1.1 Transgenic Animal Studies	160
5.1.2 Analysis of Patient Samples	161
5.2 Keratin Gene Mutations Identified in This Study	164
5.3 Clustering of Pathological Mutations	166
5.4 Keratin Gene Mutations Suggest a Structural Role for Keratins	171
5.5 The Role of Individual Residues	172
5.6 Assessing the Functional Significance of Keratin Gene Mutations	175
5.7 Keratin Filament Assembly and the Location of Keratin Mutations	178
5.8 Are Keratin Gene Mutations Responsible for all Cases of EBS and BCIE	181
5.9 Other Diseases Involving Intermediate Filament Abnormalities	182
5.10 Prospects for EB Simplex Patients	185
5.11 Gene Therapy Prospects for EB Simplex	186
5.12 Conclusion and Future Work	190
 Chapter 6. References	 192
 Appendices	 219
Appendix I Cell Culture: Materials and Methods	220
Appendix II DNA Analysis: Materials and Methods	222

Appendix III	Protein Analysis: Materials and Methods	234
Appendix IV	UK DEBRA Epidermolysis Bullosa National Register	238
Appendix V	Published Paper	240

Abbreviations

EBS	Epidermolysis bullosa simplex
EBS-WC	EBS-Weber-Cockayne
EBS-K	EBS-Koebner
EBS-DM	EBS-Dowling-Meara
EBS-R	Recessive EBS
JEB	Junctional epidermolysis bullosa
DEB	Dystrophic epidermolysis bullosa
K	keratin
DMEM	Dulbecco's modified Eagle's medium
FCS	foetal calf serum
PBS	phosphate buffered saline
EDTA	ethylenediaminetetra-acetic acid
DMSO	dimethyl sulphoxide
dNTP	2' deoxynucleoside 5' triphosphate
DEPC	diethyl pyrocarbonate
TEMED	N, N, N', N' - tetramethylethylenediamine
DTT	dithiothreitol.
TAE	tris-acetate EDTA
TBE	tris-borate EDTA
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis

A	Adenine
T	Thymine
C	Cytosine
G	Guanine

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

CHAPTER 1

GENERAL INTRODUCTION

1.1 Epidermolysis Bullosa (EB)

Epidermolysis bullosa (EB) refers to a variety of inherited bullous disorders characterised by a common tendency to develop blisters of the skin, and sometimes the mucous membranes, in response to relatively minor mechanical trauma. These disorders affect all races, with approximately equal sex distribution. In general, blistering occurs from birth or infancy and continues throughout life, the severity depending on the type of EB (Lin & Carter, 1989). The name “epidermolysis bullosa” was first used by Koebner (1886) and is now known to include the three sub-forms; EB simplex (EBS), junctional EB (JEB) and dystrophic EB (DEB). These three types of EB can be readily distinguished by ultrastructural examination of a fresh blister as each has a very specific cleavage plane within or adjacent to the epidermal basement membrane, the junction between the dermis and the epidermis (Pearson, 1962).

1.2 The Human Skin

The human skin consists of an outer stratified keratinising epidermis, an underlying dermis and a sub-dermal fat layer (figure 1). The primary functions of the skin are to provide protection against physical injury, wear and tear, impermeability to the passage of chemicals and resistance to microbial infection. The epidermis is of similar thickness all over the body (75-150 μm) with the exception of the palms and soles (400-600 μm), and is composed mainly of keratinocytes with some antigen-presenting Langerhans cells, melanocytes (which produce the pigment melanin) and Merkel cells.

Keratinocytes are attached to each other by desmosomes and express cytoskeletal keratin intermediate filaments. The epidermis is divided into several layers; the basal germinative layer of keratinocytes, the stratum spinosum (several layers of polyhedral cells), the stratum granulosum (a layer of flattened cells containing keratohyalin granules) and the outermost stratum corneum (flat anucleate cornified cells).

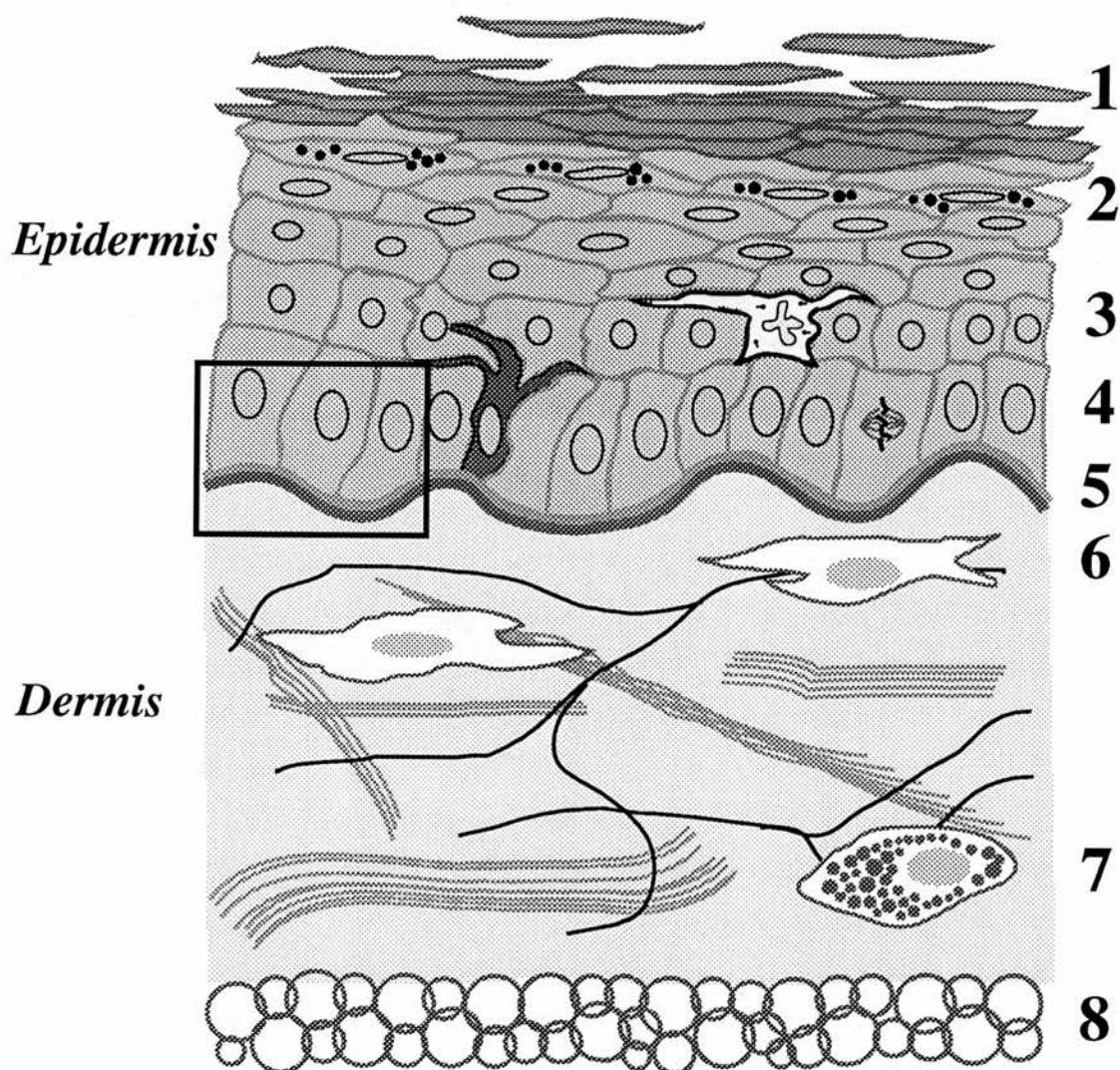


Figure 1. Diagram of the skin in vertical section (adapted from Priestley, 1993). (1) stratum corneum shedding dead corneocytes; (2) stratum granulosum layer; (3) stratum spinosum layer with Langerhans cells (white) among the keratinocytes; (4) basal germinative layer of keratinocytes with melanocyte (dark) and one keratinocyte in mitosis; (5) epidermal basement membrane consisting of two parallel layers, the upper electron-lucent lamina lucida and the lower lamina densa; (6) fibroblasts in the upper dermis among the collagen bundles, elastic fibres (branched) and glycosaminoglycans (grey background); (7) mast cell in the lower dermis; (8) subdermal fat.

Inset (figure 2) shows the level of blister formation in the three main types of epidermolysis bullosa.

The epidermis is in a constant state of turnover; as the basal keratinocytes divide some are displaced and move up through the layers to become the terminally differentiated keratinocytes of the stratum corneum that are eventually lost into the environment. Normal human epidermis is thought to completely renew itself in 45-75 days (Halprin, 1972; Bergstresser & Taylor, 1977). The underlying dermis is of variable thickness in different regions of the body and consists of a dense fibrous network of collagen and elastic fibres in an extracellular matrix of proteoglycans, fibronectin and other proteins. The principle cell types embedded in the collagen meshwork are fibroblasts and mast cells. The dermis provides strength and elasticity to the skin, support to the vascular and nerve networks, and encloses excretory and secretory glands, and keratinised appendage structures such as hair and nail.

Epidermal Basement Membrane

At the interface between the dermis and the epidermis lies the epidermal basement membrane (dermo-epidermal junction). The major epidermal cell type at this junction are the basal keratinocytes but melanocytes and Merkel cells are involved (Briggaman, 1982). Within this basement membrane there are a large number of structures, many of which, can now be distinguished by ultrastructural, biochemical and immunological criteria (Woodley & McNutt, 1992; Tidman, 1993). Some of these specialised components (keratin intermediate filaments, hemidesmosomes, anchoring filaments, lamina densa and anchoring fibrils) form a structural network to connect the cytoskeleton of the keratinocytes with the underlying dermal tissue, thereby maintaining the structural integrity of the basal keratinocytes and in turn, the suprabasal layers of the epidermis.

At the ultrastructural level, the epidermal basement membrane can be seen to consist of two parallel layers, the upper electron-lucent lamina lucida (30-40 nm thick) and the lower electron-dense, lamina densa (30-50 nm thick). Hemidesmosomes are electron-

dense structures (500-1,000 nm in diameter) which stud the basal keratinocyte plasma membrane and consist of an intracellular and extracellular portion. The intracellular electron-dense attachment plaque (20-40 nm thick) on the inner side of the basal plasma membrane within the keratinocytes serves as an insertion point for keratin intermediate filaments; exactly how the keratin filaments attach to the hemidesmosomes is unknown. Within the lamina lucida, just outside the basal keratinocyte is a narrow extracellular electron dense sub-basal dense plate. The lamina lucida contains several proteins including laminin and, entactin (Carlin *et al.*, 1981) otherwise known as nidogen (Timpl *et al.*, 1983). Some bullous pemphigoid antigen is found in the lamina lucida but the majority is localised to the hemidesmosomes. Several integrins are expressed in the epidermal basement membrane zone, for example, $\alpha 6 \beta 4$ integrin (Stepp *et al.*, 1990; Sonnenberg *et al.*, 1991) which is localised to the hemidesmosomes and may be involved in adhesion of basal cells to the basement membrane. BM600/kalinin (Rousselle *et al.*, 1991), also known as nicein (Marinkovich *et al.*, 1993) is present in the lamina lucida associated with hemidesmosomes, and is recognised by the monoclonal antibody GB3 (Verrando *et al.*, 1988). Epiligrin, also associated with hemidesmosomes (Carter *et al.*, 1991), may be the same protein as kalinin and nicein. Fine thread-like anchoring filaments (2-4 nm in diameter) cross the lamina lucida from the basal plasma membrane to the lamina densa. They are most abundant in the region of the hemidesmosomes and appear to connect the hemidesmosomes to the lamina densa (Ellison & Garrod, 1984).

Within the lamina densa a collagen network is formed by crosslinking of collagen IV molecules through disulphide bonds. Below the lamina densa, in the superficial dermis, is the sub-basal lamina fibrous zone where anchoring fibrils (30-100 nm in length) connect the lamina densa to the dermis, providing stability and support to the basement membrane. The main constituent of anchoring fibrils is type VII collagen

(Sakai *et al.*, 1986; Lunstrum *et al.*, 1986) and individual anchoring fibrils are tightly packed with a central cross-banded portion and fanning at the extremities.

1.3 Classification of Epidermolysis Bullosa

The three types of epidermolysis bullosa, EBS, JEB and DEB, are very different in prognosis, clinical appearance and pathogenesis (Bauer & Briggaman, 1993).

Classification of EB is based on the ultrastructural level of separation within the skin (figure 2), an assessment of the clinical features, and the mode of inheritance (autosomal dominant or recessive). EB simplex, a very separate condition from the junctional and dystrophic types, is the only type where, as the name implies, blistering due to lysis of the epidermis occurs. The characteristic intraepidermal blistering of EBS is due to cytolysis in the subnuclear region of the basal keratinocytes. In JEB, cleavage is through the lamina lucida of the epidermal basement membrane and in DEB the split is just beneath the lamina densa in the upper dermis (Pearson, 1962; Gedde-Dahl, 1981). The level of cleavage can be detected by indirect immunofluorescence mapping of monoclonal antibodies to basement membrane antigens, of known locations, within the epidermal basement membrane. In EBS, bullous pemphigoid antigen, laminin, and type IV collagen are all located on the blister floor; in JEB, bullous pemphigoid antigen is mainly seen on the blister roof with laminin and type IV collagen on the blister floor and in DEB all three basement antigens are located in the blister roof (Hinter *et al.*, 1981). Within the three main types further subdivisions, mainly on the basis of varying clinical features, have resulted in the classification of at least 20 subtypes of EB; the most common forms are summarised below.

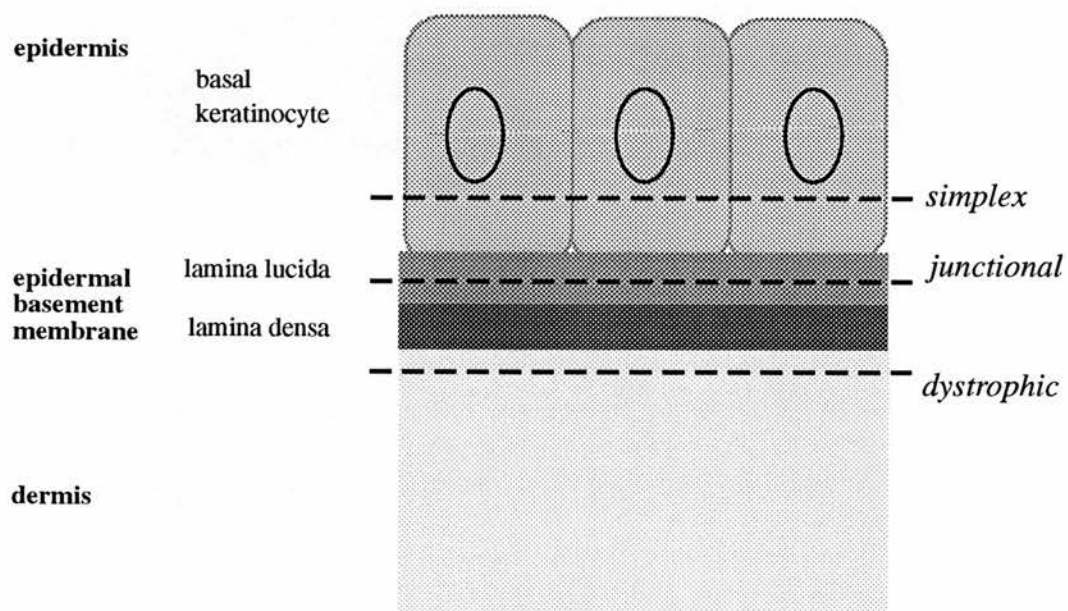


Figure 2. Inset from figure 1, to show the specific level of blister formation within or adjacent to the epidermal basement membrane for each of the three main types of epidermolysis bullosa.

Epidermolysis Bullosa

EB simplex

intraepidermal blistering
non-scarring
autosomal dominant inheritance
onset at birth or during early childhood

<i>EBS Weber-Cockayne</i>	blistering predominantly of the hands and feet
<i>EBS Koebner</i>	generalised blistering
<i>EBS Dowling-Meara</i>	herpetiform clustering of blisters blistering can be severe at birth tonofilament clumping in the basal keratinocytes

Junctional EB

blisters develop within the lamina lucida
non-scarring blisters, but atrophy may develop
mucosal membranes may be involved
nail dystrophy
autosomal recessive inheritance
onset at birth

<i>JEB Herlitz</i>	may be lethal severe, widespread blistering oral lesions, anaemia, growth retardation a quantitative and qualitative defect of the hemidesmosomes
<i>JEB non-lethal</i>	blisters result in atrophic scars good prognosis

Dystrophic EB

blisters develop just beneath the lamina densa
scarring, milia formation, nail dystrophy
mucosal membranes may be involved
onset at birth or early infancy

<i>Dominant DEB</i>	dominant inheritance reduced number of anchoring fibrils
<i>Recessive DEB</i>	recessive inheritance generally more severe than the dominant form, oesophageal involvement mitten-like scars of the hands and feet reduced or absent anchoring fibrils

Diagnosis at birth is confusing due to the variation in severity between individuals and within subtypes. Some severe EBS cases may present clinically as dominant DEB and mild recessive DEB could resemble EBS (Fine, 1988). Other blistering conditions such as bullous congenital ichthyosiform erythroderma (BCIE) and congenital herpes simplex virus infection may also have to be excluded (Lin & Carter, 1989).

1.4 Prevalence of EB

Although EB is well characterised and classified into at least 20 different subtypes, very little has been documented about the epidemiology of the disease. More information is now becoming available with the establishment of national EB registries in several countries. Studies so far have found the frequency of the three main types of EB to vary between countries, but the numbers obtained are probably all under estimates as many mild cases go undiagnosed.

A study in Oxford, England (Davison, 1965) estimated the frequency of all dominant types together to be 1:50,000 and recessive forms to be 1:300,000. Gedde-Dahl (1971) estimated the prevalence of all types of EBS in Norway to be 24/million and 1.4/million for dominant DEB and 6.6/million for recessive DEB, with a more recent estimate of 5/million for the recessive dystrophic type (Gedde-Dahl, 1978). In Finland (Kero, 1984) identification of 40 EB families over a 10 year period revealed a much lower incidence of recessive DEB compared to the Norwegian data. In 1980 data from South Africa estimated the prevalence of EB to be 2.82/million (Winship, 1990). In Japan (Inaba *et al.*, 1989) EBS was estimated at 2.9-4/million, JEB at 0.15-0.2/million, dominant DEB at 1.1-1.5/million and recessive DEB at 1.5-2.1/million with the total number of EB patients estimated to be more than 670 in 1983. Analysis of the records in Croatia over 27 years estimated the overall prevalence of EB at 9.56/million (Pavicic *et al.*, 1990) with a very low incidence of EBS (unless many mild cases go undiagnosed) but a high incidence of recessive DEB. A recent report by McKenna *et*

al. (1992b) estimates the one year prevalence of new cases diagnosed in Northern Ireland to be 1.4/million per year (0.9 EBS, 0.03 JEB, 0.3 DEB/million/year) and the prevalence of all forms is 32/million (EBS 28, JEB 0.7, DEB 3/million).

A national EB registry was begun by the Dystrophic Epidermolysis Research Association (DEBRA) in the UK in 1991. This was based on the American National EB Register established in 1986 (Carter & Caldwell-Brown, 1990), which by 1992 had recorded 1,799 patients (Lin & Carter, 1992a), and the South African National Register (Winship, 1990). Data for the registry, from affected individuals and their families, collected in the form of a questionnaire (with medical and social sections) together with a clinical examination will provide information on epidemiology, clinical symptoms, treatment, physical independence, economic and social impact of the disease, prevalence, morbidity, mortality, geographic distribution and frequency of the different subtypes (McGrath *et al.*, 1992a). The information will provide a resourceful database for EB sufferers and, scientists involved in research into the pathogenesis of EB. Data for the Scottish section of the UK register has been collected by Dr. H. M. Horn and Dr. M. J. Tidman (Dermatology Department, Edinburgh Royal Infirmary) and is now nearing completion (see Appendix IV). To date, 230 EB sufferers have been identified in Scotland giving a prevalence of 46/million. Preliminary data (Horn *et al.*, 1994) shows the most common form in Scotland to be EB simplex (67%) then dystrophic (37%), with very few cases of junctional EB (1%).

1.5 EB-like Disorders in Animals

Blistering disorders with characteristics similar to EB have been reported in animals, including an EBS-like disease in collie dogs (Scott & Schultz, 1977); an inherited skin disease with bullae formation and shedding of the epithelium from oral mucosa and exposed parts of the skin in Suffolk and South Dorset Down sheep in New Zealand (Alley *et al.*, 1974); a congenital bovine epidermolysis resembling EBS in man

(Bassett, 1987); a disease resembling JEB in a toy poodle (Dunstan *et al.*, 1988); a mechanobullous disease in 2 Belgium foals (Kohn *et al.*, 1989) and a severe congenital mechanobullous disease in sheep (Bruckner-Tuderman *et al.*, 1991). Genetic, clinical, ultrastructural and immunochemical findings from this last report suggest that this recessive dermolytic blistering disorder corresponds to severe recessive DEB in humans, raising the possibility that it could be used as an animal model to investigate the human disorder. However, at present, there is no adequate animal model for studying the pathogenesis of EB simplex.

1.6 Epidermolysis Bullosa Simplex (EBS)

In EBS, cytolysis in the subnuclear region of the basal keratinocytes results in blister formation. The suprabasal layer of the epidermis becomes separated from the dermis and fragments of the basal keratinocyte are left at the base of the blister (Pearson, 1962); perinuclear swelling can be seen by electron microscopy (Lin & Carter, 1989). This differs from the structure of an ordinary friction blister where the split occurs higher up in the epidermis, in the spinous cells (Hunter *et al.*, 1974). The hemidesmosomes and anchoring fibrils appear to be normal (Lin & Carter, 1992b) but in the most severe form of EBS, Dowling-Meara (DM), the tonofilaments (keratin filaments) of the basal keratinocytes appear in characteristic clumps.

While in general EBS is considered to be the mildest form of EB, and in some cases may even go undiagnosed, blistering can be severe, particularly at birth in the EBS-DM subtype. For some patients, routine activities such as walking, sports activities, typing, and ironing may be difficult due to recurrent blisters on the feet and hands. Some patients report improvement of the disease with age but this could be partly due to avoiding activities known to induce blister formation. Blisters heal without cutaneous scarring and any mucosal membrane involvement is usually mild and restricted to the mouth (Wright *et al.*, 1988). Nail dystrophy, although not normally

associated with EBS, does occur in some cases (Fine, 1988) and corneal involvement is very rare but has been reported (Grank & Baden, 1980). EBS is normally inherited as an autosomal dominant trait, although there are rare cases of recessive EBS.

The most common types of EBS are:

EBS Weber-Cockayne (EBS-WC)

The name arose from a case of 'recurrent bullous eruption of the feet' described by Weber (1926) and from two dominant families described by Cockayne (1938) with blistering of the feet. Since 1957 it has been used to describe a blistering disorder predominantly affecting the hands and feet due to cytolysis within the basal keratinocytes, that is inherited in an autosomal dominant manner (Wesner, 1957). Data from the establishment of national EB registries in the UK and in the US suggests that this is the most common form of EBS. Blisters develop in early or late childhood, often when a child is learning to walk, but in some cases the disorder may not be apparent or diagnosed until much later as more severe friction, such as strenuous physical activity, may be required to induce blisters (Fine, 1986). The blisters are induced by trauma from routine minor daily physical activities but blisters can also develop spontaneously. As well as on the hands and feet (figure 3a, b), blisters may occur at other friction points, for example, around the neck due to tight collars, around the waist, or on the inner thigh from riding a bicycle. The blisters sometimes bleed but heal without scarring or milia formation, and callouses may develop over areas of recurrent blistering (Vaidya *et al.*, 1991). Hyperhidrosis of the feet is common and there may be mild hyperkeratosis of palms and soles (Pearson, 1988). There is a marked seasonal variation, with more severe blistering (and more common secondary infections) occurring in the warmer summer months, and for some patients it can be disabling enough to require the use of a wheelchair.



Figure 3a. Blister on dorsum of foot in child with EBS-WC.



Figure 3b. Blisters on palm of child with EBS-WC

EBS Dowling-Meara (EBS-DM)

Dowling and Meara (1954) described a disorder present in four unrelated children of 3-7.5 years of age, who at birth or in early infancy had developed blisters in a herpetiform pattern. Originally misinterpreted as a subepidermal recessive disorder, this was later recognised as an intraepidermal autosomal dominant disease (Anton-Lamprecht *et al.*, 1979). The level of cleavage is often only a few hundred nanometers above the epidermal basement membrane, making the distinction from subepidermal blistering difficult by light microscopy (Anton-Lamprecht & Schnyder, 1982; Buchbinder *et al.*, 1986). The Dowling-Meara variant is generally considered to be the most severe form of EBS; blistering may be extensive at birth and the disorder can be fatal in the first few months. Early diagnosis by electron microscopy is therefore important to enable appropriate care in these first critical months. Dowling-Meara is inherited in an autosomal dominant manner, although many cases are sporadic. Blisters occur in herpetiform clusters on the trunk and proximal extremities and heal with minor scarring; milia may occur (figure 4a, b, c). Oral blistering is common, and the teeth and oesophagus may be affected. There is often progressive hyperkeratosis of the palms (figure 4d) and soles; nails may be dystrophic or shed (Haber *et al.*, 1985; Pearson, 1988; Fine *et al.*, 1991). There is not the same marked seasonal variation as in EBS-WC but there are reports that blistering improves during periods of fever and with age (Kero & Niemi, 1986).

Histologically, EBS-DM differs from the other forms of EBS with abnormal clumping of the tonofilaments within basal keratinocytes (figure 5a, b) preceding basal cell cytolysis and intraepidermal blister formation (Anton-Lamprecht & Schnyder, 1982; Tidman *et al.*, 1988; Ishida-Yamamoto *et al.*, 1991; McGrath *et al.*, 1992b).



Figure 4a. Blistering on foot of a baby with EBS-DM.



Figure 4b. Typical pattern of blistering on the thigh of a child with EBS-DM (same child as 4a).



Figure 4c. “Herpetiform” clustering of blisters in an adult with EBS-DM, with subtle background hyperpigmentation.



Figure 4d. Palm of the same subject as figure 4c showing an extreme degree of palmar hyperkeratosis.

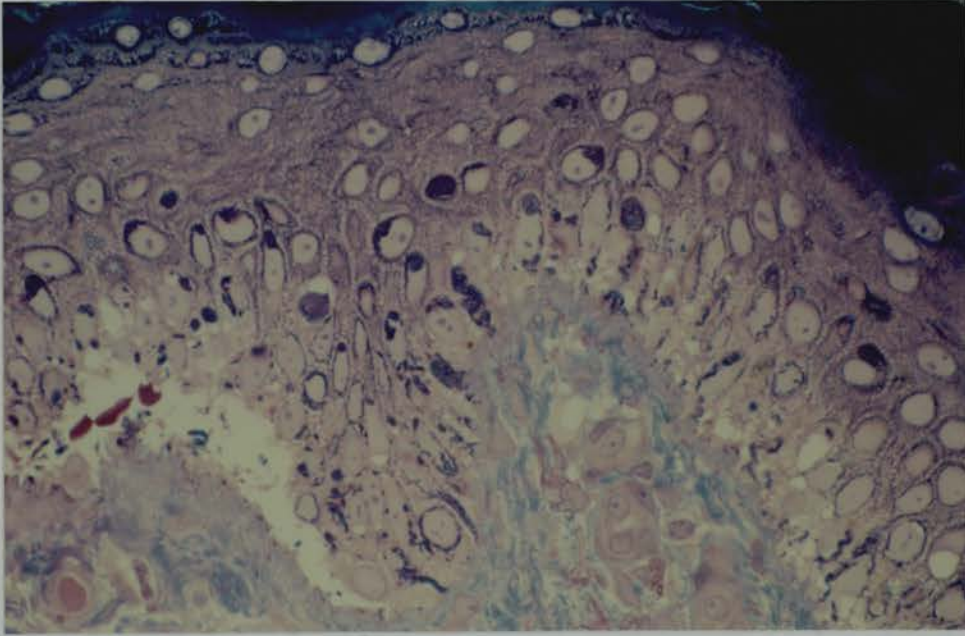


Figure 5a. Photomicrograph of semi-thin resin-embedded section of peri-lesional skin from the same subject as 4c, demonstrating fragments of epidermis in the floor of the early blister and the characteristic tonofilament clumping of EBS-DM (Huber stain).

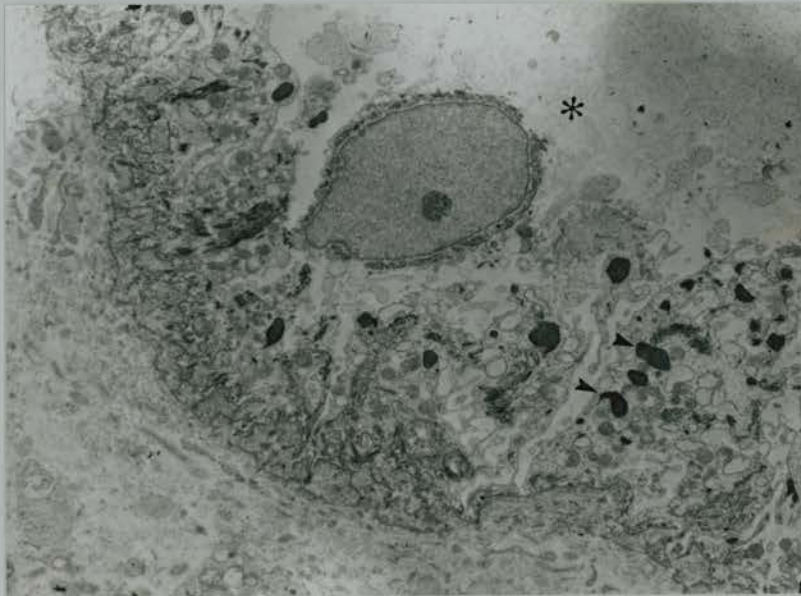


Figure 5b. Electron micrograph showing cytolysis of a basal keratinocyte (*) and clumping of the tonofilaments (shown by the arrows) in a blister from the larynx of a patient with EBS-DM. Magnification x 3600. (Photographs kindly provided by Dr. M. J. Tidman).

There are clinical and histological similarities between EBS-DM and another dominantly inherited blistering disorder of the skin, bullous congenital ichthyosiform erythroderma (BCIE), also referred to as epidermolytic hyperkeratosis (EH), figure 6 (Phillips & Baden, 1993). The trauma induced blisters of BCIE are distinguished from those of EBS-DM by the level of blistering within the epidermis. In EBS-DM blisters develop in the basal keratinocytes in comparison to the suprabasal blistering of BCIE. Hyperkeratosis is generally more localised in EBS-DM than in BCIE.



Figure 6. Elbow of subject with BCIE, demonstrating characteristic hyperkeratosis and background xerosis.

EBS Koebner (EBS-K)

The original cases described by Koebner (1886) developed blisters mainly on the feet and hands but the name is now used to describe a subtype similar to EBS-WC but with generalised blistering on all parts of the body and in the oral cavity (Haber *et al.*, 1985). However, there is often no clear clinical distinction between the EBS-WC and EBS-K

as blistering in many EBS-WC classified individuals is not restricted to the feet and hands. The friction-induced blisters of EBS-K develop at birth or in early infancy (Fine, 1986) and heal without scarring, although secondary bacterial infections are a common problem. There may be mild to moderate hyperkeratosis of soles and palms and often hyperhydrosis of the feet. The nails may show mild involvement (Pearson, 1988). There is a seasonal variation, like EBS-WC, with more severe blistering in warm weather.

Less common types of EBS include:

EBS Ogna

Characterised by seasonal (summer), non-scarring serous blisters of hands and feet that develop in infancy or in early childhood (Haber *et al.*, 1985). Blisters are frequently small and haemorrhagic. This subtype is distinguished from EBS-WC by the tendency for generalised bruising of the skin (Fine, 1986). Genetic linkage analysis has shown close linkage of this phenotype to the erythrocyte enzyme, glutamic pyruvic transaminase, at the locus EBS1, which is on the long arm of chromosome 8 (Olaisen & Gedde-Dahl, 1973).

EBS with Mottled Pigmentation

Described by Fischer and Gedde-Dahl (1979) as a variant of the dominantly inherited EBS-K. Generalised non-scarring blisters develop at birth or during early infancy. At birth the skin also has a mottled appearance due to a pigmentary abnormality, which fades with age to become barely recognisable in adults. Other associated characteristics include skin atrophy on the trunk and extremities of adults giving a premature aged appearance, mild bruising of the legs, longitudinally curved nails and hyperkeratosis of palms and soles (Haber *et al.*, 1985). One affected member of the original family described showed no mottled pigmentation, this syndrome may therefore be due to two

different genetic defects rather than a combined expression of a single mutant gene. Although quite rare, similar cases have been described with variable additional clinical features (Boss *et al.*, 1981; Medenica-Mojilovic *et al.*, 1986; Bruckner-Tuderman *et al.*, 1989; Coleman *et al.*, 1993).

Recessive EBS

Several families have been reported with an autosomal recessive form of EBS that may be associated with a neuromuscular disease (muscular dystrophy or myasthenia gravis; Niemi *et al.*, 1988; Kletter *et al.*, 1989). Severe cutaneous blistering develops from birth or early infancy. Other characteristics not normally associated with EBS but occurring in these individuals are atrophic scarring, milia formation, nail dystrophy, anaemia and growth retardation. Whether this form of EBS is a rare autosomal recessive genetic disease with both the skin and muscular manifestations being the effects of a single mutant allele or whether different genetic defects are closely linked and co-inherited is unclear (Fine *et al.*, 1989c).

Another form of recessively inherited EBS, which is associated with high mortality, has been reported in a large family (10 of 13 affected members died before two years of age). Typical EBS lysis of basal keratinocytes results in generalised blistering particularly of the hands, feet, knees and elbows, developing at or shortly after birth. Blistering is more frequent in the summer, anaemia is common and the oral mucosa is often mildly affected (Salih *et al.*, 1985). Another similar case has recently been documented in a patient from Saudi Arabia (Abanmi *et al.*, 1994).

There are also several reports of typical EBS Weber-Cockayne like disorders that have been transmitted as an autosomal recessive rather than as a dominant trait (Fine *et al.*, 1989b; Hovnanian *et al.*, 1993).

Rare Subtypes of EBS

Other rare and unusual forms of EBS also exist, demonstrating the complexity of the disease. Some of these may eventually justify classification as separate subgroups but are still grouped with the rest at present due to the small number of cases identified, while others may be variants of previously identified subgroups. They include Kallin syndrome, characterised by blistering of the feet and hands similar to EBS-WC, together with anodontia, brittle hair, nail disorders and probably transmitted as an autosomal recessive trait (Gamborg-Nielsen & Sjolund, 1985), Kindler syndrome (Kindler, 1954; Alper *et al.*, 1978; Bordas *et al.*, 1982), EBS associated with severe ulcerative colitis (Smith *et al.*, 1993) and a case of blistering predominantly of the feet and hands but with tonofilament clumping, thereby combining the clinical features of EBS-WC and EBS-DM (Eisenberg *et al.*, 1986).

The following three subgroups are often classified under EBS but it is debatable as to whether they are distinct disorders. In EB superficialis the level of skin cleavage is higher in the epidermis, just beneath the stratum corneum, a cleavage site usually associated with peeling skin syndrome (Fine *et al.*, 1989a). EBS-Bart (Bart *et al.*, 1966; Smith & Cram, 1978) is an autosomal dominant disorder characterised by congenital localised absence of the skin (on the lower extremities) together with EB-like blistering of the skin and mucous membranes, and nail deformities. This local congenital absence of skin has been associated with junctional and dystrophic as well as the simplex form of EB (Bart, 1970; Skoven & Drzewiecki, 1979; Wojnarowska *et al.*, 1983; Butler *et al.*, 1986; Kanzler *et al.*, 1992). EBS Mendes da Costa is transmitted as an X-linked recessive disorder and blisters develop on the extremities during the first two years of life, although, unlike most forms of EBS the blisters occur spontaneously and are not trauma induced (Mendes da Costa & van der Valk, 1908; Woerdeman, 1957; Gedde-Dahl & Anton-Lamprecht, 1983; Haber *et al.*, 1985).

1.7 Treatment of EB Simplex

There is no specific treatment as yet for EBS. The principle aim is to avoid factors known to induce blistering such as tight clothing, humid environment, sweating and adhesive tapes (McGrath *et al.*, 1992b), to prevent secondary infections and to encourage healing. Opening of fresh blisters with a sterile needle accelerates healing by preventing the blisters spreading due to fluid build up. Antiseptics, antibiotics, and protective moist non-adherent dressings should be used to cover lesions and changed regularly. Dressings should be applied carefully and held in place with outer soft ones rather than with tape which might induce blistering. Synthetic temporary skin substitutes can be used to cover exposed areas (Sagi *et al.*, 1988).

Several agents tested for their ability to control blistering have shown some degree of success but without justifying regular use in the treatment of EBS. Those tested include the following: 20% aluminium chloride hexahydrate (Tkach, 1982; Jennings, 1984; Younger *et al.*, 1990), topical glutaraldehyde (DesGrosseilliers & Brisson 1974), 5% topical bufexamac (Fine & Johnson, 1988), 5HT₂ antagonists (Bonnetblanc & Bouquier, 1986; Tadini *et al.*, 1993) and the retinoid, isotretinoin (Andreano & Tomecki, 1988).

Genetic counselling should be provided for affected individuals and relevant members of their family as it is important that they know and understand the genetic risks. The natural history of the disease should be discussed, for example that EBS-DM generally improves with age (while other diseases such as recessive DEB deteriorate). The fact that there can be variability in severity within members of the same family should be pointed out. The different forms of EB are inherited in different ways and have different prognoses and therefore the actual likelihood of transmission should be explained. In an autosomal dominant trait the offspring are 50% at risk of inheriting the disease. If a child, with a dominant type of EB is born to unaffected parents, as the

result of a spontaneous or new mutation, then the parents' further offspring are not at an increased risk as the mutation is assumed to be a one time event. However, offspring of the affected child are 50% at risk of inheriting EB. Autosomal recessive traits, where both parents are heterozygote carriers for the abnormal gene, carry a 25% recurrence risk with each pregnancy (Sybert & Holbrook, 1992). Early accurate diagnosis of an affected child is necessary particularly in the severe, recessive or spontaneous types as it is difficult to offer parents reliable counselling for future pregnancies if a child dies before a correct diagnosis is made.

Prenatal diagnosis is not normally carried out on pregnancies at risk from EBS except possibly for EBS-DM (Holbrook *et al.*, 1992). Diagnosis or exclusion has been performed for JEB (Anton-Lamprecht, 1981; Lofberg *et al.*, 1983; Blanchet-Bardon *et al.*, 1984), dominant DEB (Blanchet-Bardon *et al.*, 1987) and recessive DEB (Anton-Lamprecht *et al.*, 1981). The most reliable method of prenatal diagnosis of EB is by transmission electron microscopy of foetal skin obtained by foetoscopy (Rodeck *et al.*, 1980). In the case of EBS-DM, the skin is examined for the presence/absence of keratin filament aggregates in the basal keratinocytes and for separation of the epidermis from the dermis through the basal cells.

DEBRA (Dystrophic Epidermolysis Bullosa Research Association), an association set up by EB patients and their families, provides invaluable support, advice and counselling for families and patients affected by all forms of EB. DEBRA newsletters, meetings and, sponsorship of recent scientific research has increased public awareness of EB and of the special needs of those affected. This is important to prevent further unfortunate cases where lesions typical of EB have been misdiagnosed as child abuse (Eby, 1988, Winship & Winship 1988).

1.8 Pathogenesis of EB Simplex

When this study was started there were three possible hypotheses to explain the mechanism of intraepidermal blister formation of EBS. Either there was an excessive proteolytic activity within or around the basal cells, perhaps associated with lysosomal fragility (Pearson, 1971), or there was a mutant temperature-sensitive structural protein within the basal cells (Gedde-Dahl, 1978; Haneke & Anton-Lamprecht, 1982), or a component of the keratinocyte cell membrane was abnormal (Fine & Griffith, 1985). Investigations, by independent research groups, have concentrated on these theories to identify the underlying cause of the intracellular blistering of EBS.

1.9 Theories on the Cause of EB Simplex

1.9.1 A Proteolytic Enzyme Abnormality

Evidence that an enzyme abnormality was directly involved in the pathogenesis of EBS came from various studies but differing enzyme abnormalities were suggested.

Although in EBS the blisters are intraepidermal, studies revealed some unexpected abnormalities in the dermal fibroblasts. Decreased levels of galactosylhydroxyllysyl glucosyltransferase (a sugar transferase involved in collagen biosynthesis) were found in serum, skin and cultured fibroblasts of some, but not all, affected members of an EBS-K family (Savolainen *et al.*, 1981). Despite the fact that this abnormality was present in some unaffected members and was not detected in a further two EBS-K families it was suggested that, in some families, there could be close linkage between the gene coding for the enzyme and that coding for EBS-K.

Sanchez *et al.* (1983) reported a decrease of a gelatin-specific neutral-metallo protease in cultured fibroblasts from 3 EBS-K families and in 6 of 13 EBS-WC patients. They proposed that this defect might be a marker for EBS-K and that EBS-K and EBS-WC could be closely related genetic disorders. This defect could represent a pleiotrophic effect of the gene for EBS or it could be genetically linked to the EBS gene. However,

further examination of gelatinase expression in 6 patients from 3 EBS-K families by Winberg and Gedde-Dahl (1986) did not support these findings, leading to the conclusion that reduced gelatinase expression from dermal fibroblasts is not uniformly a marker for EBS-K. The baseline level of gelatinase activity in conditioned medium from fibroblasts from a group of Scandinavian EB patients was recently measured (Winberg & Gedde-Dahl, 1992). High and low activity levels occurred throughout the different EB groups, but the high activity levels were more frequent among EBS, particularly in EBS-WC. In comparison to the previously reported reduced activity in EBS-K (Sanchez *et al.*, 1983), these results suggested that EBS-K and EBS-WC could be caused by different gene abnormalities. The increase in gelatinase activity may be due to either altered expression of gelatinase or to an allelic variant of this enzyme with increased specific activity. Support for possible close linkage between an EBS gene and a gene responsible for increased gelatinase expression requires further investigations, but it seems unlikely that dermal elements and the epidermal basement membrane are directly involved in the pathogenesis of EBS.

Various proteases can be used experimentally to separate the epidermis and the dermis, with the level of separation dependent on the protease used. For example, trypsin separates the epidermis through the basement membrane, and collagenase separates the epidermis beneath the basement membrane (Takamori *et al.*, 1988). Investigations into several skin diseases, including bullous pemphigoid (Naito *et al.*, 1982) and pemphigus (Schiltz *et al.*, 1979, Morioka *et al.*, 1981), claimed that it was possible to produce separation of normal skin identical to that seen *in vivo*, when the skin was incubated with blister fluid or sera from an affected individual. Such studies led to the proposal that proteases, specific for each skin disease may be involved in inducing blister formation, with the possibility of a similar mechanism being involved in the blister formation of EB.

Takamori *et al.* (1983) and Manabe *et al.* (1984) investigated the possibility that EB blister fluid contained specific proteases that could promote skin separation at a specific level, ie. EBS blister fluid could induce intraepidermal separation while DEB blister fluid could induce a subepidermal split. Normal human skin explants were cultured *in vitro* with EB blister fluid (by the method of Sarkany *et al.*, 1965). The explants were examined at 12, 24, 48, and 72 h intervals by light and electron microscopy, and stained by routine methods (haematoxylin and eosin, PAS and silver stain). They demonstrated that fresh blister fluid from EBS patients, when cultured with normal human skin *in vitro*, induced intraepidermal separation of the skin. Cytolysis of the lower part of the epidermis began after 24 h in culture, resulting in fully developed blisters after 48 h in culture with similar histological features to those of EBS. In a similar way, fresh blister fluid from recessive DEB induced subepidermal blistering in normal skin after 48 h in culture. Skin explants cultured with burn and normal suction blister fluid showed no histologic changes. Similar experiments with blister fluid from JEB individuals (Matsumoto & Hashimoto, 1984, 1986; Matsumoto *et al.*, 1985) resulted in cleavage within the lamina lucida of normal human skin. It was proposed that the blister fluid from the different types of EB contained selective properties, possibly enzymes, responsible for inducing blister formation in specific cleavage planes in normal human skin.

Further studies (Takamori *et al.*, 1985) involved pretreatment of EBS and recessive DEB blister fluid with various protease inhibitors, including heat (60°C for 30 min), trypsin digestion, EDTA, soybean trypsin inhibitor (a serine protease inhibitor), N-ethylmaleimide (a thiol protease inhibitor), pepstatin and α_2 macroglobin, prior to organ culture. The addition of certain inhibitors prevented skin separation enabling the authors to further characterise the blister inducing factor(s). When EBS blister fluid was pretreated with heat (60°C for 30 min), trypsin digestion or N-ethylmaleimide, no intraepidermal separation was observed, but EDTA, soybean trypsin inhibitor,

pepstatin and α_2 macroglobin had no inhibitory affect, leading to the proposal that this blister inducing factor was a neutral thiol protease. Pretreatment of recessive DEB blister fluid with trypsin digestion, EDTA, soybean trypsin inhibitor, N-ethylmaleimide, α_2 macroglobulin and heating to 60°C for 30 min, all inhibited blister formation, but dialysis was not inhibitory. The recessive DEB blister-inducing factor was proposed to be a non-dialysable high molecular weight protein, which could include a combination of a metallo-protease (eg. collagenase), a serine protease or a neutral thiol protease. Further investigations indicated that this recessive DEB blister-inducing factor was produced by fibroblast cells (Ikeda *et al.*, 1985). On the basis of these results, a small clinical trial was carried out with a serine protease inhibitor, camostat mesylate, for the treatment of recessive DEB, which resulted in some reduction in the number of new blisters (Ikeda *et al.*, 1988).

Despite the apparent success of Takamori *et al.* (1985) and Matsumoto & Hashimoto (1986) in demonstrating specific cleavage of normal skin with blister fluid from different types of EB, Fine *et al.* (1986) were unable to reproduce these findings. In similar studies with blister fluid from EBS, JEB, and DEB, they reported that while some blister fluid did induce blisters with the predicted cleavage planes, when the results were examined in a blinded manner the groups could not be distinguished from each other (Fine *et al.*, 1986). They concluded that this skin organ culture technique was an unsuitable *in vitro* model for the study of EB and their data did not support the hypothesis of selective protease release in EB. It should be noted that all the above studies involved a relatively small number of patients.

1.9.2 Structural Abnormalities of the Keratinocyte Cell

Membrane

Another line of investigation involved possible abnormalities of the keratinocyte cell membrane, such as a decreased, absent or biochemically altered structural component (Fine & Griffith, 1985). Clinically normal skin from EBS patients (6 EBS-WC, 2 EBS-K) was stained by immunofluorescence with 8 fluorescein-labelled affinity-purified lectins and the pattern of the lectins binding to the keratinocyte cell membrane recorded. With peanut agglutinin all the EBS samples showed irregular and focally granular epidermal membrane staining, in contrast to the uniform membrane staining observed with JEB, DEB and normal skin controls. The EBS samples showed some staining with the other 7 lectins. Keratin cytoskeletal staining with anti-keratin antibodies AE2 and AE3 was normal for EBS-WC and EBS-K and therefore, did not indicate any keratin abnormality. The EBS keratinocyte cell membrane was proposed to have abnormal glycosylation of a glycoprotein (or glycolipid) that was specific to peanut agglutinin, which could result in abnormal interactions between epidermal cells leading to increased cell fragility. Two other studies supported the idea of structural abnormalities in the keratinocyte cell membranes of EBS skin. Sakamoto *et al.* (1988) reported a lack of binding of soybean agglutinin in the cell membranes of keratinocytes in basal and spinous cells of EBS skin, from blister edges or mechanically traumatised areas. JEB, DEB and control skin showed similar binding patterns to each other but distinct from EBS. A cytofluorometric study (Hachisuka *et al.*, 1990) revealed a lower intensity of several lectins (*lectin Ricinus communis agglutinin*, peanut agglutinin and soybean agglutinin) bound to the basal cells of EBS than to normal controls, and there were no differences in JEB and DEB cells.

1.9.3 A Defective Structural Protein in the Basal Keratinocytes

The first indication that an abnormality in a basal cell structural protein could underlie at least some forms of EBS was from ultrastructural studies of the most severe variant, Dowling-Meara. Abnormal clumping of the tonofilaments (keratin filaments) was observed in the basal keratinocytes in association with blistering (Anton-Lamprecht *et al.*, 1979, Anton-Lamprecht & Schnyder, 1982, Niemi *et al.*, 1983; Tidman *et al.*, 1988). In later studies, tonofilament clumping was observed in some non-lesional, as well as in peri-lesional skin and in adnexal epithelia including sweat ducts, outer hair root sheaths and sebaceous glands (Ishida-Yamamoto *et al.*, 1991; McGrath *et al.*, 1992b). These clumped keratin filaments were labelled by antibodies to the basal keratins, K5 and K14, rather than for other epithelial keratins. Abnormal round bodies that stained for these basal keratins were also present in cultured keratinocytes of EBS-DM patients (Ishida-Yamamoto *et al.*, 1991). This characteristic clumping of the keratin filaments has been reported in further cases of EBS-DM (Kates *et al.*, 1992) and is used as a marker in diagnosis of the disease. Originally it was not clear whether the tonofilament clumping was a primary abnormality or a secondary effect due to cytolysis. However, as tonofilament clumping had been reported to precede cytolysis and blister formation (Anton-Lamprecht *et al.*, 1979) and was later reported to occur in the absence of cytolysis, in non-lesional skin and in cultured keratinocytes, it was suggested that tonofilament clumping was a primary event due to specific basal keratin abnormalities rather than as a consequence of blistering. However, what actually caused this clumping of the tonofilaments and the resulting cell fragility was unknown.

Keratin filaments are a type of intermediate filament and are thought to be the major structural proteins within the epidermal cells. Any abnormality making the cytoskeleton less resilient was a potential candidate for the cause of blistering in EBS. Possible defects leading to increased cell fragility included structural or charge changes of the

keratin molecules due to amino acid mutations, an abnormal post-synthetic modification such as phosphorylation or proteolysis, or an abnormal interaction with a keratin filament associated protein or some other unknown factor (Ishida-Yamamoto *et al.*, 1991).

During the course of this project the genetic defect in several families affected by EBS-DM (Coulombe *et al.*, 1991a; Lane *et al.*, 1992) and in one EBS-K family (Bonifas *et al.*, 1991b) was identified. Point mutations found, in affected members of these families, in the genes encoding the basal keratins (K5 and K14) were proposed to be the underlying cause of the disease. However the question remained: were defects in the basal cell keratins also responsible for the relatively mild Weber-Cockayne form of EBS? The basal keratinocytes of both the Koebner and Weber-Cockayne variants of EBS normally show few, if any, ultrastructural abnormalities (Haneke & Anton-Lamprecht, 1982) and, when immuno-labelled with antibodies to keratins K5, K14, K1 and K10, a similar staining pattern to that of normal human skin was observed (Ishida-Yamamoto *et al.*, 1991). Abnormalities had, however, been reported in the organisation of keratin filaments in cultured keratinocytes from two Koebner patients (Kitajima *et al.*, 1989; one of these patients has since been reclassified as EBS-DM, Ishida-Yamamoto *et al.*, 1991) and in one case of EBS-K where there was a lack of tonofilaments in the basal cells in both normal and affected skin of a young child (Ito *et al.*, 1991). Although few, if any, ultrastructural abnormalities are observed in the basal keratinocytes of the Weber-Cockayne variant, it was possible that similar, but more subtle alterations to the keratin cytoskeleton, to those identified in EBS-DM and EBS-K, were responsible for this milder variant. Perhaps the effect of a keratin mutation resulting in relatively mild disruption to the keratin filament network is only observed when the keratinocytes are in context of the host tissue.

Genetic linkage analysis provided the first evidence that a keratin abnormality could underlie EBS-WC. This technique is used to predict where, and on what chromosome, an abnormal gene responsible for an inherited disease lies. Polymorphisms, two or more alleles at a given locus (Mischke *et al.*, 1990), occur naturally within the human genome either as single nucleotide substitutions or as several base pairs (deletions or insertions). Most polymorphisms (95%) have no effect on the function of the gene but they can be used to map genetic diseases by tracing their pattern of inheritance through several generations of a family with multiple affected members with the disease. A DNA polymorphic marker and a disease gene that are close together on the same chromosome are more likely to be inherited together as they are less likely to become separated by random recombination events than loci that are further apart. Co-segregation of a marker with the disease in every affected member of the family indicates that the 2 loci are “linked”; this can be expressed as a LOD score (logarithm of the odds in favour of linkage) (Ott, 1974).

Genetic linkage analysis of several EBS-K and EBS-WC families had supported tentative linkage of the disease with markers on the long arm of chromosome 1, although no candidate genes had been identified (Hauge, 1962; Mulley *et al.*, 1984; Humphries *et al.*, 1990). More recent studies though, showed linkage of EBS-K and EBS-WC to either chromosome 12 or to chromosome 17. Bonifas *et al.* (1991a) noticed that similarities, such as autosomal dominant inheritance and temperature-sensitive cellular fragility, existed between EBS-K, EBS-WC and some heritable erythrocyte disorders, for example pyropoikilocytosis. Since red blood cells can become fragile due to mutations in the cytoskeletal proteins, they investigated, by genetic linkage analysis, the possibility that an abnormality in a keratinocyte cytoskeletal protein, ie. keratin, could underlie these variants of EBS. Keratins are known to be clustered on chromosome 17 (type I keratins) and on chromosome 12 (type II keratins) (Lessin *et al.*, 1988; Romano *et al.*, 1988), therefore, DNA

polymorphisms near these keratin gene clusters were used to compare the inheritance of EBS with these polymorphic markers. Two families affected by EBS were studied (Bonifas *et al.*, 1991a). In one, the EBS-Koebner phenotype showed linkage to chromosome 17 (type I keratins) and a point mutation in keratin 14 was subsequently identified (Bonifas *et al.*, 1991b). In the second family, with the Weber-Cockayne variant of EBS, the disease was linked to loci that map near the type II, keratin 5 gene, on chromosome 12. These findings provided strong evidence that a defect in a basal keratin gene, K5 or K14, could also underlie some cases of EBS-WC, in addition to EBS-DM and EBS-K.

1.10 Intermediate Filaments

The cytoskeleton of all eukaryotic cells consists of three main networks; microfilaments (5-7 nm diameter), microtubules (25 nm diameter) and intermediate filaments (10 nm diameter). Actin and associated proteins of the microfilament network and, the microtubule system, are involved in various cellular events including cell division, contraction, orientation and polarisation and anchorage. The function of the third network, the intermediate filaments, has largely been undefined but there is now increasing evidence to suggest that this network has a structural role acting as a cytoskeleton within cells. Studies on vertebrate cells have provided most of the data on intermediate filament proteins, although there is now an increasing number of reports of intermediate filament like proteins in lower animals, fungi, plants and unicellular organisms. Intermediate filament proteins are classified into at least six types on the basis of sequence homology, tissue specificity and immunological activities. In the cytoplasm intermediate filaments form a complex array of filaments from the nucleus to the plasma membrane and in the nucleus intermediate filaments, present as nuclear lamins, are on the inner surface of the nuclear membrane.

Types of Intermediate Filament Proteins

Type	IF Protein	mw kD	Cell Type
Type I	Keratin	40-56.5 kD	Epithelia
Type II	Keratin	53-67 kD	Epithelia
Type III	Vimentin	57 kD	Mesenchymal cells
	Desmin	53kD	Muscle cells
	Glial fibrillary acidic protein (GFAP)	50 kD	Glial cells and Astrocytes
	Peripherin	57 kD	Peripheral neurons
Type IV	Neurofilament proteins		
	NF-L	62 kD	
	NF-M	102 kD	Neuronal cells
	NF-H	110 kD	
	α internexin	65kD	
Type V	Lamin proteins		All nucleated cells
	A-type lamins: A, C	70 kD, 60kD	
	B-type lamins: B1, B2	67 kD	
Type VI	Nestin	240 kD	Neuronal stem cells

Nestin is usually classified as a type VI intermediate filament, but some consider it to be a type IV intermediate filament.

The chromosomal locations of various intermediate filaments have been identified. Human keratin intermediate filaments are located at two defined sites within the genome, the type I keratins to chromosome 17q12-q21 (Lessin *et al.*, 1988; Romano *et al.*, 1988; Rosenberg *et al.*, 1988) and the type II keratins to chromosome 12q11q-14 (Lessin *et al.*, 1988; Romano *et al.*, 1988; Popescu *et al.*, 1989; Rosenberg *et al.*, 1991). An exception is the type I keratin, K18, which is located on chromosome 12 (Waseem *et al.*, 1990;) therefore, both members of the K8 and K18 pair are on the

same chromosome. In humans, vimentin is located on chromosome 10 (Quax *et al.*, 1985; Ferrari *et al.*, 1987), desmin on chromosome 2 (Quax *et al.*, 1985), GFAP on chromosome 17, NF-L on chromosome 8 (Hurst *et al.*, 1987) and NF-H on chromosome 22 (Lieberburg *et al.*, 1989). Vimentin, desmin and GFAP are each encoded by a single gene and the more complex neurofilament (NF) family by three different genes. Lamins A and C are encoded by a single gene, and lamin B by a separate gene.

1.11 Keratins

Keratins, the most complex family of intermediate filaments, are a multigene family encoding at least 30 polypeptides (Coulombe, 1993). These water-insoluble proteins are present in most vertebrate epithelia. They form a cage-like array of filaments around the nucleus and out towards the desmosomes where they are thought to be attached through desmoplakin proteins (Green *et al.*, 1990). Keratins are numerically classified; each is a separate gene product that can be identified by two-dimensional gel electrophoresis, isoelectric point, molecular weight and tissue distribution. Proteolytic mapping of keratins has shown that they differ only to a limited extent in their primary sequences (Fuchs & Green, 1978). Most keratins exist as one copy but there are two genes for keratins K6 and K2. K6a and K6b differ in the 3' non-coding sequence, but it is not known whether they are differentially expressed (Tyner *et al.*, 1985). The two keratin K2 genes show limited sequence homology and are expressed in different tissues, K2p in oral and K2e in epidermal tissues (Collin *et al.*, 1992b). Since the original classification by Moll *et al.* (1982), of 19 human keratins another human keratin has been identified, keratin 20, that is expressed in intestinal epithelia (Moll *et al.*, 1990). Approximately 30% of the protein in basal cells is keratin and in fully differentiated cells the majority of the protein (approximately 85%) consists of keratin (Fuchs, 1990).

Keratins are subdivided, mainly on the basis of sequence homology, into two types; the acidic type I keratins, K9-K20, with a range in molecular weight from 40-56.5 kD (pKi 4.5-5.5) and the larger, neutral-basic (53-67 kD, pKi 5.5-7.5) type II keratins, K1-K8 (Fuchs *et al.*, 1981, Moll *et al.*, 1982). Keratins coassemble as obligate heterodimers (Eichner *et al.*, 1986) consisting of equimolar amounts of a type I and a type II keratin, and are expressed in a tissue and differentiation specific manner. Each type II keratin of the pair is approximately 8 kD larger than the type I keratin (Cooper *et al.*, 1985) and in the epidermal and corneal keratins, expression of the type II keratin (K1 or K3) precedes that of the type I keratin (K10 or K12 respectively). Simple epithelial cells, ie. those with a free luminal surface and in contact with the basal lamina, express keratins K8 and K18. The basal cells of stratified epithelia (those in contact with the basal lamina but without a free luminal surface) express the primary keratins K5 and K14. Suprabasal cells (those which have no contact with the basal lamina) express small amounts of the primary keratins but predominantly express a pair of tissue specific secondary keratins (Lane & Alexander, 1990).

In normal human skin, as keratinocytes migrate from the basal layer towards the stratum corneum, the primary keratins (K5 and K14) are down regulated and the suprabasal cells, now committed to terminal differentiation, express predominantly the secondary keratins, K1 and K10. In certain body regions smaller amounts of other secondary keratins are expressed; keratin 9 (type I) is expressed specifically in palmar and plantar epidermis (Knapp *et al.*, 1986; Moll *et al.*, 1987) and the type II keratin, K2, is expressed in suprabasal cell layers, at a late stage of differentiation and after keratin 1 (K2e in epidermal and K2p in oral tissues; Collin *et al.*, 1992a). Keratin 15 is expressed in some stratified squamous epithelia along with keratins, K5 and K14 (Fuchs, 1988) and, keratin 19 which has been described as "promiscuous" is expressed alongside many other keratins. In hyperproliferating skin, during wound healing or in

cultured keratinocytes, keratins K6, K16 and K17 are expressed in addition to the tissue specific secondary pair.

Keratin Expression in Different Epithelial Cell Types

	Type I	Type II
Simple epithelial cells	K18 (45 kD)	K8 (52 kD)
	K19 (40 kD)	K8 (52 kD)
Intestinal epithelia	K20 (46 kD)	K8 (52 kD)
Basal keratinocytes	K14 (50 kD)	K5 (58 kD)
	K15 (50 kD)	
Simple and some stratified epithelia	K17 (46 kD)	K7 (54 kD)
Hyperproliferative keratinocytes	K16 (48 kD)	K6 (56kD)
Non-cornifying stratified epithelia (oesophagus)	K13 (51 kD)	K4 (59 kD)
Corneal differentiation	K12 (55 kD)	K3 (64 kD)
Cornified epithelia	K10 (56.5 kD)	K1 (68 kD)
		K2 (65.5 kD)
	K9 (64 kD)	
	K11 (56 kD)	

Keratins present in hair epithelial cells differ from epithelial cytokeratins; at least eight different ones have been classified (Heid *et al.*, 1986).

Pseudogenes exist for some keratins, including K8, K14, K16, K17, K18 and K19 (Kulesh & Oshima, 1988; Rosenberg *et al.*, 1988; Savtchenko *et al.*, 1988a,b; Troyanovsky *et al.*, 1992). Pseudogenes are related in sequence to their functional gene, with exons and introns in the usual positions, but due to deleterious mutations gene expression is prevented and they cannot be translated into functional proteins (Lewin,1985).

Keratin gene expression is controlled so that in any epithelia tissue, equimolar amounts, of the specific type I and type II keratins are produced. Regulation of gene expression is thought to be at transcription (Steinert *et al.*, 1985) or post-transcription (Tyner & Fuchs, 1986), but there is probably some control at the post-translational level, to regulate groups of proteins. Various factors including retinoids (vitamin A derivatives), environmental elements, and tumor promoters (Steinert & Freedberg, 1991) can control keratin gene expression.

1.12 Structure of Intermediate Filaments

From investigations involving quantitative methods of structural analysis (scanning transmission electron microscopy and nuclear magnetic resonance), and from primary sequence analysis, it has been established that all intermediate filament protein subunits have a similar basic structure. This consists of a central α -helical rod of conserved secondary structure of approximately 310 amino acids, flanked by a non-helical amino (head) and a carboxy (tail) terminal domain (figure 7). Different types of intermediate filaments share approximately 25-40% sequence identity in the α -helical rod domain (Hanukoglu & Fuchs, 1983). Within the same type, keratins share 50-99% sequence homology of the α -helical domain with the non-helical end termini providing the major variations between keratin pairs (Fuchs, 1988).

The central rod domain consists of heptad repeats, $(a, b, c, d, e, f, g)_n$ with hydrophobic amino acids at the first (*a*) and fourth (*d*) residues. This results in an α -helical structure due to the apolar residues on one side of the helix (Steinert & Freedberg, 1991). Glycine and proline residues are rarely found in the α -helical regions as they often destabilise coiled coils. Most of the variation in sequence in the α -helical domains is at the second (*b*), third (*c*) and sixth (*f*) residues of the heptad, which are located on the outer surface of the coiled coil (Conway & Parry, 1988).

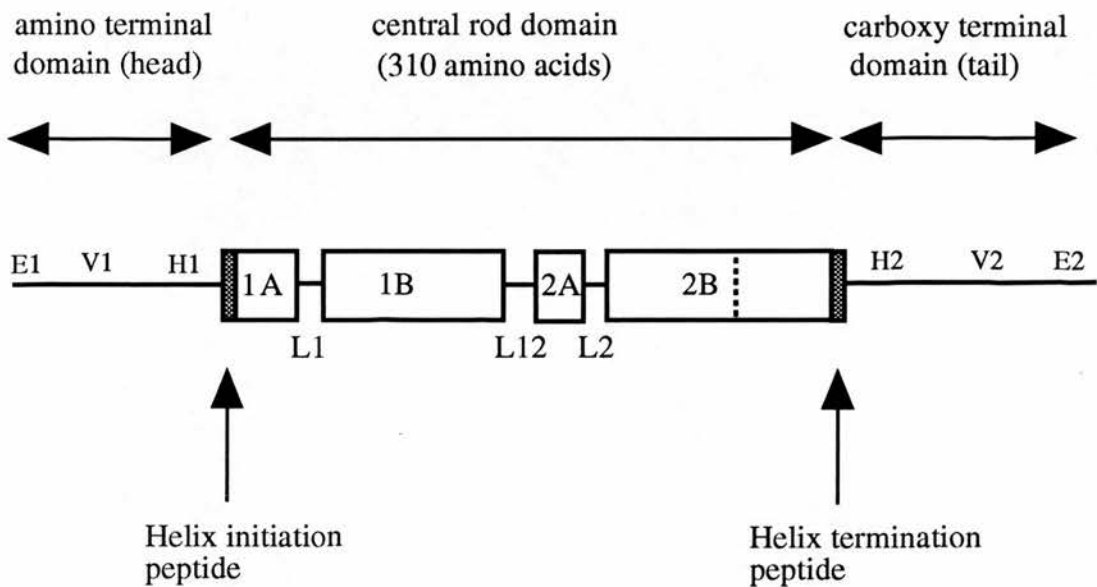


Figure 7. Diagrammatic representation of a keratin molecule. The boxes indicate the four conserved α -helical subdomains (1A, 1B, 2A and 2B) that are connected by the non-helical linker regions (L1, L12 and L2). The shaded areas represent the highly conserved helix initiation peptide at the amino terminal and the helix termination peptide at the carboxy terminal of the rod domain. The "stutter" in the 2B helix is shown by a dashed line. The non-helical amino terminal and carboxy terminal flank the central α -helical rod domain. Note, the H1 domain in the type I keratins is very small and there is no H2 domain in the type I keratins.

The α -helical rod is interrupted by three linker regions. These domains, which only share 5-20% sequence identity between intermediate filaments, are thought to provide some flexibility to the rod as they do not contain any heptad repeats and therefore cause a break in the coiled coil at these points. The L1 linker joins the 1A and 1B α -helical domains to form segment 1, the L2 linker joins the 2A and 2B α -helical domains to form segment 2 and the L12 linker connects the 2 segments together at 1B and 2A. The L1 and L12 linker domains vary slightly in length among intermediate filaments, being of 8-14 residues and 16-19 residues respectively and are predicted to be non-helical, except in type V lamin intermediate filaments where they are predicted to be α -helical (Parry *et al.*, 1986). In comparison, the L2 linker is conserved in length (8 residues) for all types of intermediate filaments. The L2 linker also shows a high degree of homology within and between different intermediate filament types leading to the prediction that this domain could be α -helical even though it lacks the characteristic heptad repeats of the other α -helical domains (Conway & Parry, 1988). Towards the centre of the 2B domain there is a further break in the heptad repeat due to a reversal in polarity which creates a stutter or discontinuity.

The variation in charge between the type I and type II keratins is mainly due to different numbers of basic amino acids in this 2B domain (Steinert *et al.*, 1984). Each of the domains, 1A, 1B, 2A and 2B, are predicted to be α -helical and are conserved in length between intermediate filaments, 35, 101, 19, and 121 residues respectively. The total length of the rod domain is approximately 45-48 nm, with each of segments 1 and 2 being approximately 22 nm in length (Steinert *et al.*, 1985). Type V intermediate filaments differ from the other types with a slightly longer central rod domain, approximately 350 residues, due to an additional 42 residues at the end of the 1B domain (Conway & Parry, 1988). The two extreme ends of the rod domain are the most highly conserved regions between intermediate filaments with 90-99% sequence identity (McCormick *et al.*, 1991). These are known as the helix initiation peptide

(HIP), residues 8-20 of the 1A domain, and the helix termination peptide (HTP) which includes the last 30 residues of the 2B domain (Steinert *et al.*, 1985).

Outside the rod domain, the non-helical terminal domains are highly variable in sequence and size between intermediate filaments and are responsible for most differences in size and properties of intermediate filaments. These domains are thought to be located on the surface of the filaments (Steinert *et al.*, 1983) and to be involved in inter-filament associations and interactions with cellular structures. The specific function of these domains is determined by the properties of their subdomains.

The type II keratins contain a highly conserved domain on either side of the rod domain, the H1 of 36 residues, at the amino terminal and the H2 of 20 residues, at the carboxy terminal (figure 7). The type I keratins only have a small HI domain (Steinert, 1993). These extra domains explain the mass difference between the type I and type II keratins. Both type I and type II keratins have a V1 and a V2 subdomain which, for each keratin, vary in sequence and size (0-130 residues). The size of these variable domains increases in relation to the increasing complexity of the epithelium and they often contain tandem repeats rich in glycine and/or serine residues which increase the insolubility of the molecule (Steinert *et al.*, 1985). The V1 and V2 sequences are proposed to have a secondary structure based on Ω loops due to the high number of glycine and serine residues and the probable location on the surface of the protein (Stewart, 1990). At the ends of the amino and carboxy termini of type I and II keratins are the highly charged (basic) E1 and E2 domains. Keratin 19 is an exception; it does not have a non-helical carboxy terminal but instead, the α -helical rod domain is extended by 13 amino acids beyond the helix termination peptide (Bader *et al.*, 1986; Stasiak *et al.*, 1989). Other types of intermediate filaments also possess some of these subdomains, type III intermediate filaments have E1, H1 and H2 domains and type IV

intermediate filaments have E1, H1, H2, V2 and E2 domains (Steinert & Freedberg, 1991).

Several epidermal keratins (K1, K4, K5, K10) are known to be polymorphic due to the small differences in their electrophoretic migration rates observed by SDS polyacrylamide gel electrophoresis analysis (Mischke & Wild, 1987). Korge *et al.* (1992b) reported that the polymorphism in keratin 10 was due to variations in size from 401 to 515 base pairs in the V2 subdomain of the non-helical carboxy terminus, which could result in a difference of up to 38 amino acids at the protein level. Sequence analysis of keratin 4 revealed three alleles due to differences in the V1 subdomain (Wanner *et al.*, 1993). In keratin 5 a single nucleotide substitution in the H1 subdomain, resulting in an amino acid change from glycine to glutamic acid, was identified as the allelic variation (Wanner *et al.*, 1993).

1.13 Evolution of Intermediate Filaments

Intermediate filament proteins are related by their structure and the amino acid sequence homology of their central α -helical domain (Oshima, 1992). They are thought to have evolved from lamins (Weber *et al.*, 1989) which are the most highly conserved of intermediate filaments and the only type to be expressed throughout all eukaryotes. Type I, II and III intermediate filaments share high conservation of seven or eight intron positions. Most of these introns are in regions encoding the rod domain, at points corresponding to the start of heptad repeats in the coiled coil (Krieg *et al.*, 1985). However, the structure of the type IV and VI intermediate filaments is very different, with only neurofilament-H (NF-H) having one intron that is conserved with the other intermediate filament genes (Lees *et al.*, 1988). This suggests that duplication of the original gene resulted in two gene lineages, one for type I, II and III intermediate filament genes and another for the neuronal filament genes.

1.14 Intermediate Filament Assembly

Approximately 10,000-20,000 subunits are required to form a 10 nm intermediate filament. Intermediate filaments must encode all their structural information necessary for assembly as no additional proteins are required (Stewart, 1993). The first stage in the assembly process is the formation of a dimer (figure 8, 1). Two monomer chains align in parallel and in axial register to form a coiled coil α -helical molecule of the form described by Crick (1953). Hydrophobic residues at the first (*a*) and fourth (*d*) positions in the heptad repeats results in a stripe of these residues running around the α -helical chains (Parry, 1990). This type of structure was first reported for wool keratins (Crewther *et al.*, 1978) and has since been found to be a characteristic of all intermediate filament subunits. Keratins form obligatory heterodimers requiring a type I and type II keratin, demonstrated by filament *in vitro* studies with combinations of purified subunits (Coulombe & Fuchs, 1990; Hatzfeld & Weber, 1990a; Steinert, 1990). The type III and IV intermediate filaments can form homodimers or heterodimers with other members of the type III or type IV classes respectively (Steinert *et al.*, 1982). Type V (nuclear lamins) do not heteropolymerize with other types.

The next stage in assembly of a 10 nm filament is the formation of a tetramer by the alignment of two dimers. The precise spatial arrangement of these two coiled coils is still uncertain and has been controversial. Steinert (1993) suggests from cross-linking experiments that there are three possible ways that keratin dimers could align in an antiparallel way. At the two-molecule level the two molecules may be staggered so that either the 1B domains are aligned or the 2B domains are aligned (figure 8, 2b, c). At the three- and four-molecule level of assembly the two molecules can be aligned in register (figure 8, 2a). Chemical cross linking studies also suggest that desmin dimers (type III intermediate filaments) are arranged in an antiparallel and partially staggered way with the 1B domains overlapping (Geisler *et al.*, 1992). It is possible, therefore,

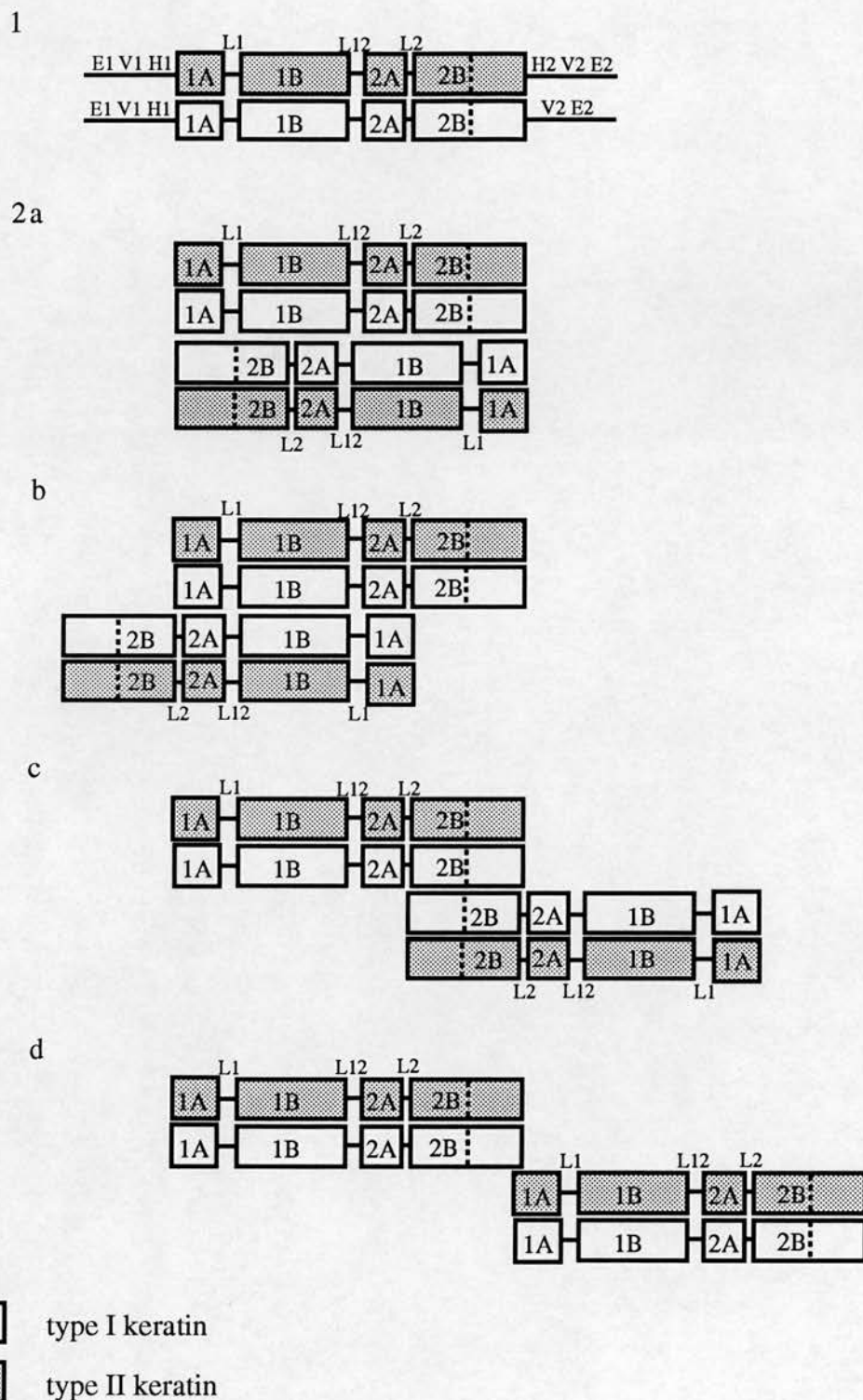


Figure 8. Diagram to show the proposed alignment of monomers and dimers during keratin filament assembly (adapted from Steinert 1993). (1) The first stage in keratin filament assembly is the alignment of a type I and a type II keratin in register and in parallel to form a coiled coil heterodimer; (2) The predicted modes of antiparallel alignment of two dimers; in register (a), staggered (b, c). (2d) The predicted overlap of two neighbouring molecules, aligned in either the (2b) or (2c) arrangement when lying in the same direction. (The non-helical terminal domains have been omitted).

that all types of intermediate filaments are assembled in a similar way. If the keratin intermediate filaments align in either of the staggered antiparallel ways suggested, it is predicted that, since the axial repeat length of 45nm is less than the length of each keratin molecule (46 nm) there is an overlap between neighbouring molecules lying in the same direction (figure 8, 2d). The last ten residues of the 2B domain of one molecule would overlap with the first ten or so residues of the 1A domain of the adjacent molecule.

Tetramers polymerize, both laterally and longitudinally, to form 2 to 3 nm protofilaments and when two of these align a 4.5 nm protofibril is formed. The intertwining of protofibrils results in a complete intermediate filament consisting of 24-40 monomer chains in cross section depending on the intermediate filament protein. Keratin intermediate filaments usually contain 32 monomer chains, in cross section (Steven *et al.*, 1983). The precise arrangement of the keratin filaments to form a three dimensional structure is not yet clear.

Post-translational Modification of Intermediate Filaments

All intermediate filaments may undergo some form of post-translational modification. The major modification is by phosphorylation and intermediate filaments are substrates for several protein kinases. Phosphorylation of keratins occurs mainly on the serine and threonine residues in the non-helical amino and carboxy terminal domains (Steinert, 1988). Phosphorylation and dephosphorylation may control structural changes and the regulation of the dynamic properties of intermediate filaments (Eriksson *et al.*, 1992). Evidence suggests that in general, phosphorylation inhibits intermediate filament assembly and promotes disassembly (Stewart, 1993). Other processes by which intermediate filaments may be modified include proteolysis, crosslinking by transglutaminases, acetylation and glycosylation (Steinert, 1988).

Intermediate Filament Associated Proteins (IFAPs)

The interaction of intermediate filaments with intermediate filament associated proteins (IFAPs) adds further complexity to the network. IFAPs, usually expressed in a cell specific manner, may be involved in the control or assembly of filaments and may be responsible for the functional diversity among intermediate filaments. Among the more defined IFAPs are the histidine-rich family of filaggrins, which are expressed in keratinising epithelium and cause the aggregation of keratin filaments in cornified epidermal cells (Foisner & Wiche, 1991). Plectin, another IFAP, with a widespread tissue and cell type distribution, binds vimentin, some keratins, glial fibrillary acidic protein (GFAP), neurofilaments (NF), and lamin B via the central α -helical domains (Foisner *et al.*, 1988) and interacts with intermediate filaments in a phosphorylation dependent manner (Foisner & Wiche, 1991). Plectin may also have a general cytoplasmic crosslinking function and may connect intermediate filaments with the other cytoskeletal filament systems, microtubules and microfilaments. Other IFAPs include trichohyalin, loricrin, a major constituent of keratinocyte cell envelopes, desmoplakins I and II of desmosomes (Steinert, 1993), synemin and paranemin.

1.15 Dynamic Properties of Intermediate Filaments

Until relatively recently, due to their low solubility and stability *in vitro*, intermediate filaments were generally considered to be relatively stable structures. However, recent microinjection studies have shown rapid incorporation of newly synthesized subunits into existing intermediate filament networks, thereby demonstrating their previously unrecognised dynamic properties (Mittal *et al.*, 1989; Vikstrom *et al.*, 1989; Miller *et al.*, 1991; Vikstrom *et al.*, 1992). Microinjection of biotinylated type I keratins into PtK2 epithelial cells (potoroo rat kidney epithelial cells that express both a simple epithelial keratin and vimentin network) resulted in the formation, within 2 h, of an extensive biotinylated keratin intermediate filament like network (Miller *et al.*, 1991). This dynamic process is controlled by phosphorylation and dephosphorylation

(Steinert, 1993). Further studies by Miller *et al.* (1993) to investigate the earliest steps in the assembly of keratin subunits into keratin filaments demonstrated that, before the formation of filaments, aggregates of the incorporated type I (biotinylated) keratin with the endogenous type II keratin are formed. Fluorescent spots of both type I and II keratins were observed within 1 min of injection, detected by double-labelled immunofluorescence. In epithelial cells a state of equilibrium is thought to exist between keratin subunits and polymerised keratin intermediate filaments, providing an exchangeable pool of keratin within the cell.

1.16 Deletion and Point Mutagenesis Studies of Keratins

Many studies have been carried out involving truncated intermediate filaments to determine which sequences are essential and, whether they differ, for normal intermediate filament assembly and network formation. Most of these studies have concentrated on the role of the variable non-helical terminal domains. In keratins, a series of amino and carboxy terminal deletion mutants of keratin 14 were constructed and either transfected into epithelial or squamous cell carcinoma cell lines to examine their effect on filament network formation, or assessed for their ability to form 10 nm filaments *in vitro*. Deletion of the entire amino or carboxy terminal domain of keratin 14 did not prevent the incorporation of the mutant protein into the keratin network of the cultured epithelial cells nor 10 nm filament assembly of the mutant keratin 14 with wild type keratin 5 *in vitro*. However, deletions including even small portions of the rod domain were deleterious to the filament network: the larger the deletion the more severe the disruption and collapse of the filament network and deletions to the amino terminal were more disruptive than those to the carboxy terminal. Mutants lacking the highly conserved Leu-Leu-Glu-Gly-Glu motif at the carboxy terminal of the rod domain (figure 9) could assemble into 10 nm filaments *in vitro* with only slight aberrancies and were incorporated, but caused collapse of the endogenous filament

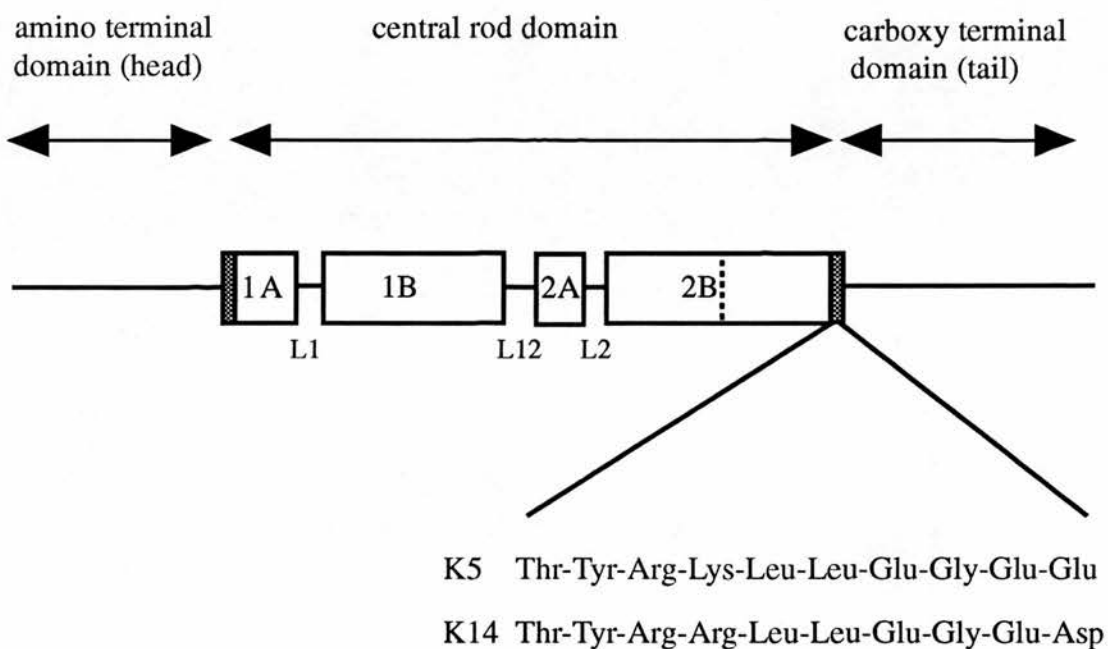


Figure 9. Diagrammatic representation of a keratin molecule to show the highly conserved sequence motif that is present in nearly all intermediate filaments at the carboxy terminal of the rod domain. The fourth and fifth residues of the sequence shown are the least conserved in this motif, although the variations at these sites are conservative.

network of transfected epithelial cells (Albers & Fuchs, 1987, 1989; Coulombe *et al.*, 1990).

Recently, Wilson *et al.* (1992) defined further the roles of the non-helical domains in filament assembly *in vitro*, using headless, tailless, headless/tailless and Arg/Lys-Leu-Leu-Glu-Gly-Glu truncated mutants of both keratins, K5 and K14. With mutations in both keratins it was possible to determine whether, in the previous studies, the normal keratin 5 had compensated for deletions in the keratin 14 mutant and, whether the domains of keratins K5 and K14 have an equal role in filament assembly. Deletion of the head domain of keratin 5 resulted, when combined with wild type keratin 14, in the formation of short and branched filaments with an irregular diameter. In contrast, deletion of the head domain of keratin 14 did not result in any major disruption to filament assembly. As predicted, when the head domains were deleted from both keratins, K5 and K14, aberrant structures of shortened filaments with irregular widths were formed. It was suggested that the head domain of keratin 5 is necessary for elongation and proper lateral alignment of filament subunits, but it is not known whether this is a common feature of the head domains of all type II keratins.

Surprisingly when the highly conserved Arg/Lys-Leu-Leu-Glu-Gly-Glu motif was deleted from both keratins, K5 and K14, filaments were still formed but were of varying diameter (10-24 nm). It was proposed that this motif may be required for the lateral alignment of subunits during 10 nm filament assembly but is not essential for all of the interactions in filament assembly. Filaments (10 nm) still assembled when the tail domains were deleted from both keratins, K5 and K14. These mutants were more sensitive than the wild type to changes in ionic strength suggesting that the tail domains may be involved in filament stability. It may not be surprising that the tail domain of at least one member of the pair is not necessary for filament formation (Bader *et al.*, 1986) as the human keratin K19 is naturally tailless but can still form filaments with K8.

Several other studies, *in vivo* and *in vitro*, have investigated the role of the terminal domains of the simple epithelial keratins (Hatzfeld & Weber, 1990b; Lu & Lane, 1990; Bader *et al.*, 1991). Together with the findings of similar deletion studies in other types of intermediate filaments (Traub & Vorgias, 1983; Gill *et al.*, 1990; Eckelt *et al.*, 1992), the central α -helical rod domain has been demonstrated to be critical for proper filament functioning in all types of intermediate filaments. However the importance of the non-helical terminal domains varies between the type of intermediate filament. These domains may have additional functions when in the natural host cell and on the surface of the intermediate filament. They may be involved in regulating intermediate filament assembly (Kouklis *et al.*, 1991) or be important for interactions between intermediate filaments and other organelles, for example, the nucleus (Georgatos & Blobel, 1987).

Point mutagenesis studies to determine the roles of individual amino acids, in comparison to deleting whole sections of the keratin molecule, have so far have been restricted to the α -helical rod domain. Amino acids have been shown to differ in their role in filament assembly and network formation, depending on the amino acid and the location of the residue in the intermediate filament. Even quite subtle amino acid substitutions in the highly conserved ends of the rod domain can produce deleterious effects to filament formation, whereas some internal substitutions are tolerated with only minor disruption to the integrity of the filament network. Hatzfeld and Weber (1991), demonstrated that single amino acid substitutions in the conserved Thr-Tyr-Arg-Lys-Leu-Leu-Glu-Gly-Glu sequence of a type I (K18) or type II (K8) keratin affected filament assembly *in vitro* with the formation of large aggregates, although dimer and tetramer formation were apparently unaffected. Letai *et al.* (1992), altered the α -helicity by introducing proline mutations throughout the rod domain of keratin 14, and examined the effect both *in vivo* (in transfected epithelial cells) and on 10 nm filament assembly *in vitro*. Existing proline and glycine residues from the L1 and L12

linker domains were also substituted to favour an α -helical conformation, and subtle mutations were introduced near the carboxy end of the rod domain. Proline residues introduced in the centre of the rod only resulted in mild aberrations to network formation and filament assembly compared to the more severe disruption observed when proline residues were substituted into the highly conserved terminal ends of the rod domain. Removal of existing proline residues did not result in any dramatic effects on the filament network and the mutants could still assemble into filaments although they tended to form aggregates.

1.17 Aim of Project

The aim of this project was to investigate the mechanisms of blistering in the most common form of EBS, the Weber-Cockayne variant.

Excessive proteolytic activity within or around the basal cells or the presence of a mutant temperature-sensitive structural protein within the basal cells (Pearson, 1971; Gedde-Dahl, 1978; Haneke & Anton-Lamprecht, 1982) had been suggested as possible causes. During the course of this project, discoveries by Coulombe *et al.* (1991a), Bonifas *et al.* (1991b) and Lane *et al.* (1992) indicated that single point mutations in either of the basal cell keratins, K5 or K14, might be primarily responsible for the increased cell fragility of the keratinocytes in the more severe forms of EBS. It appeared that an enzyme abnormality was unlikely to be directly involved in the pathogenesis of the disease, although it could be a secondary factor.

Attention was therefore, then concentrated on locating mutations, by DNA sequence analysis, in the suspected defective genes, those encoding the basal keratins, K5 and K14. Five very informative families, affected by EBS Weber-Cockayne, were studied. The aim was to identify keratin mutations and to establish whether there was any correlation between the clinical severity of the disease and the location of the mutation within the keratin molecule.



CHAPTER 2

ENZYME STUDIES

The possibility that an enzyme abnormality was directly involved in the pathogenesis of EBS was investigated by two different approaches.

2.1 Effect of Neutral Blister Fluid on Skin Explants

Reports by Takamori *et al.* (1983, 1985) suggested that specific factors, present in the blister fluid of EB patients, could induce blistering of normal skin with the level of skin separation determined by the origin of the blister fluid (see review Chapter 1, 1.9.1).

The aim was to carry out similar experiments, initially using blister fluid from EBS patients, to establish whether these results could be reproduced and to determine whether this system was a suitable *in vitro* model for the study of EB.

Method

Blister fluid was collected from fresh blisters (less than 24 h old) from patients with EBS and from suction blisters made on normal volunteers. Preliminary studies to assess the reliability and reproducibility of this organ culture technique involved incubation of the skin in Dulbecco's Modified Eagle's Medium (DMEM) only. Normal human skin (foreskin or breast skin) was cut into pieces (approximately 2 mm x 2 mm, 0.5 mm thick) and placed dermis side down on wax-edged sterile lens paper (Sarkany *et al.*, 1965). The lens paper rafts were floated on DMEM medium in 24-well tissue culture plates and incubated at 37°C in 5% CO₂: 95% air. Explants were removed at 12, 24, 48 and 72 h intervals, snap frozen in liquid nitrogen and cryostat sections cut onto polylysine (0.01%) coated slides. The sections were stained with 1% toluidine blue or Giemsa, mounted in DPX mounting medium and examined by light microscopy.

Results

Some foreskin sections showed dermal-epidermal separation after 48-72 h in culture with medium only and with normal suction blister fluid. Breast skin appeared to be

more suitable for this type of experiment since no dermal-epidermal separation was observed after 72 h of culture in medium.

These preliminary findings suggested that, in agreement with Fine *et al.*, 1986 (see review Chapter 1, 1.9.1), this is an unsuitable *in vitro* model to study any possible selective protease release in EB. My main problem however, was the practicality of obtaining enough fresh blister fluid (from out-patients) for controlled studies and to be able to pretreat some of the blister fluid with various inhibitors prior to culture.

2.2 Protease Activity in Cultured Keratinocytes and Whole Skin

A number of proteases and their inhibitors are present in the human skin and are involved in the regulation of many biological functions. Proteases present in epidermis are involved in terminal differentiation with catalysis of organelles and nuclei to form the stratum corneum. The human epidermis contains an acid proteinase, cathepsin D, that hydrolyses haemoglobin, and chymotrypsin-like enzymes that are primarily of dermal origin, probably from mast cells (Fraki *et al.*, 1986). Lysosomal proteases such as cathepsin A, cathepsin B and cathepsin D are important for protein catabolism (Takamori *et al.*, 1988). Cathepsin D is a lysosomal carboxyl (acid) protease that is widely distributed in many cell types and, cathepsin B, a cysteine protease, is found in lysosomes of mammalian cells (Bajkowski & Frankfater, 1975).

The level of protease activity in cell homogenates from cultured keratinocytes and also from normal skin was investigated as a possible factor involved in the formation of blisters.

Method

Keratinocytes were cultured by the Rheinwald and Green system (see Chapter 3, 3.1) from skin biopsies obtained from clinically uninvolved skin of patients with EBS and from normal human skin as a control. Extracts were prepared from the cultured cells and also from normal, whole and separated skin (Qian *et al.*, 1989). Protease activity was measured using two universal substrates, haemoglobin and azocasein (Beynon & Bond, 1989). As the substrates underwent proteolysis, the rate of appearance of the peptides was a measure of the activity of the proteinase(s). More specific assays to measure the levels of cathepsin B (Bajkowski & Frankfater, 1975) and cathepsin D (Takahashi & Tang, 1981) were also carried out. A radial diffusion assay was investigated as an alternative method of measuring protease activity (Beynon & Bond, 1989). The coloured enzyme substrate (haemoglobin) was dissolved in agar, and cell homogenates incubated on the agar gel produced a clear zone where proteolysis occurred.

Results

Preliminary studies were carried out using foreskin keratinocyte cultures, and also extracts from whole and separated skin, to measure the production of proteolytic enzymes and to determine baseline levels of enzyme activity before measuring the levels in EBS cultures. However, very low values were obtained which could have been due to using insufficient cells for the sensitivity of the assays or to the method of extraction.

Discussion

When investigating protease activity several features need to be considered. Protease activity can be regulated by various factors, including the rate of synthesis, degradation and the interaction of the proteases with their inhibitors (Takamori *et al.*, 1988). It is necessary therefore, to consider the effect or presence of the protease inhibitor, as normal levels of a protease would conceivably induce blister formation if the amount of

protease inhibitor were reduced. Another important consideration is that in normal physiological conditions proteases are often present in a latent form. It could, therefore, be the initiating factor that is abnormal in some way (Takamori *et al.*, 1985). Further studies would require measurements of both protease activators and inhibitors in the blister fluid.

With the discovery of keratin mutations in some EBS patients (Bonifas *et al.*, 1991b; Coulombe *et al.*, 1991a; Lane *et al.*, 1992), it became increasingly unlikely that proteases were directly involved in inducing blister formation and these enzyme studies were not continued. It is possible that proteases are a secondary factor involved in the progress of blister formation and spreading in EBS.

CHAPTER 3

KERATIN STUDIES: METHODS

The general strategy for detecting keratin mutations in EBS patients involved examination of the DNA sequences of keratin 5 and keratin 14, the suspected defective genes. Basal keratinocytes, which express keratins 5 and 14, were cultured from skin biopsies obtained from non-lesional skin of EBS patients. Polyadenylated mRNA was extracted from the cells and reverse-transcribed to produce keratin 5 and keratin 14 cDNA. Genomic DNA was extracted from whole blood samples from both affected and unaffected members of a family. The cDNA and genomic DNA was PCR amplified, purified and sequenced directly to look for any discrepancies from the predicted sequences. To exclude the possibility that a mutation was an innocuous polymorphism, occurring within the normal population, and that instead it was the likely cause for the disease, genomic DNA from affected and unaffected members of the family and from 50 unrelated control samples was screened. When available genomic DNA, from both affected and unaffected members of a family, was used for genetic linkage analysis prior to DNA sequencing to predict whether the disease was linked to either of the keratin gene clusters. The type I keratins (K14) are located on chromosome 17 and the type II keratins (K5) on chromosome 12. Cytokeratins were also extracted from the cultured cells and analysed by SDS polyacrylamide gel electrophoresis (PAGE) and Western blotting to show which keratins were expressed by the keratinocytes.

Cell Culture

For recipes of media and suppliers of chemicals see Appendix I.

3.1 Primary Keratinocyte Cell Culture

Keratinocytes were cultured, from skin biopsies obtained from non-lesional skin from patients with EBS, from unaffected unrelated controls and from the TR146 squamous cell carcinoma cell line, by the Rheinwald and Green system (1975). The keratinocytes were maintained on a monolayer of mouse 3T3 feeder cells which had been treated, either with gamma irradiation or with mitomycin C, to prevent them dividing (mitomycin C disrupts microtubule formation) and overgrowing the keratinocytes. The 3T3 cells could still metabolise and produce factors that induced keratinocyte growth; they also suppressed fibroblast growth. As well as serum, a source of poorly defined growth factors, the medium required other additives (transferrin, insulin, tri-iodothyronine, epidermal growth factor, cholera toxin and hydrocortisone) to stimulate human keratinocyte cell growth. It is now possible though, with the recent development of more defined media such as MCDB 152, MCDB 153 (Tsao *et al.*, 1982; Boyce & Ham, 1983; Pittelkow & Scott, 1986) and GIBCO serum-free keratinocyte medium, to culture keratinocytes without serum or 3T3 feeder cells. The addition of bovine pituitary extract replaces the function of the feeder cells. Keratinocyte cultures can be subcultured approximately 6-10 times before the cell line dies out, unless the cells are transformed, in which case a continuous cell line is obtained.

The skin was stored in transport medium for up to 24 h at 4°C. The dermis and excess fat was removed using scissors. The skin was washed in 0.02% ethylenediaminetetraacetic acid (EDTA) in a petri dish for 5 min, cut into small pieces and incubated in 0.25% trypsin for 2 h at 37°C (shaking every 30 min) or at 4°C overnight. Excess trypsin was poured off and discarded; the skin was transferred into a petri dish and

using fine forceps the epidermis was separated from the dermis. Both the dermis and epidermis were transferred to a universal containing Dulbecco's Modified Eagle's Medium (DMEM)-10% foetal calf serum (FCS). A single cell suspension was obtained by shaking the tube vigorously, and filtering the mixture through a stainless steel mesh to remove the dermis, stratum corneum etc. The epidermal cells were centrifuged at 1,000 rpm for 5 min, resuspended in RM-medium and a sample stained with Trypan blue dye (0.4% solution in phosphate buffered saline (PBS)) to distinguish dead cells (which stain blue) from the viable epidermal cells which do not take up the dye. The cells were plated at approximately 2×10^6 viable cells/25 cm² flask onto treated 3T3 feeder cells in RM-medium and incubated at 37°C in 5% CO₂ : 95% air. The medium was changed to RM+medium (containing epidermal growth factor) 48 h after initial plating, by which time clumps of cells were beginning to flatten out, and thereafter twice a week; more feeders were added if necessary. A 25 cm² flask provided sufficient cells for mRNA and cytokeratin extractions.

Subculture of Keratinocytes

The keratinocytes were trypsinised when approximately 70% confluent or 8-10 days after plating. The medium was discarded, the cells rinsed with PBS and incubated with 2-3 ml 0.02% EDTA in PBS for 5 min at room temperature to remove the 3T3 feeder cells and any contaminating fibroblasts. By gently tapping the flask the feeder cells were dislodged and could be removed leaving the keratinocytes, which have a stronger affinity for the plastic, still attached. The keratinocytes were incubated in 2 ml 0.05% trypsin in EDTA at 37°C for 5-10 min or until all the cells were detached. The cells were washed in DMEM medium-10% FCS, centrifuged at 1,000 rpm for 5 min, resuspended in RM-medium and plated onto 3T3 feeder cells (prepared the previous day) at 5×10^5 - 1×10^6 cells/25 cm² in RM-medium. After 48 h the medium was changed to RM+medium and when the cells were about 70% confluent they were subcultured in the same way.

Cryopreservation of Keratinocytes

Keratinocytes were stored in liquid nitrogen in 90% FCS, 10% dimethyl sulphoxide (DMSO) at 2×10^6 cells/ml. When required, the cells were thawed quickly at 37°C, washed with DMEM medium-10% FCS and centrifuged at 1,000 rpm for 5 min. The cells were resuspended in RM-medium, counted and plated onto treated 3T3 feeder cells.

3.2 3T3 Feeder Cells

Swiss mouse 3T3 cells were grown in DMEM medium-10% FCS at 37°C in 5% CO₂ : 95% air and were split 1:3 or 1:5 twice a week (they can be subcultured up to 35-40 times). To subculture the cells, they were rinsed with 5 ml PBS/75 cm² flask and incubated at 37°C with 2-3 ml 0.05% trypsin in EDTA for approximately 5 min or until the cells detached. The cells were washed in DMEM medium-10% FCS, centrifuged at 1,000 rpm for 5 min, resuspended in RM-medium and seeded at 5×10^5 cells/25 cm² flask or 2×10^6 cells/75 cm² flask.

Cryopreservation of 3T3 Feeder Cells

Stocks of 3T3 cells were stored in liquid nitrogen at 2×10^6 cells/ml in DMEM medium-20% FCS with 10% DMSO. When required, the cells were thawed quickly at 37°C, washed with DMEM medium-10% FCS, centrifuged at 1,000 rpm for 5 min and resuspended in DMEM medium-10% FCS.

Treatment of 3T3 Feeder Cells

The feeder cells were treated to prevent them dividing but they were still able to metabolise, produce growth factors and look relatively healthy for about a week after treatment. Both of the following methods were used, with equal success. It was preferable to treat and seed the feeder cells, in RM-medium, 24 h before adding the

keratinocytes. This allowed the cells to attach and be checked for contamination, but both feeder cells and keratinocytes could be added to the flask at the same time.

Irradiation with Cobalt 60

The cells were trypsinised, counted, irradiated with 6,000 rads and plated out at 5×10^5 cells/25 cm² or 2×10^6 cells/75 cm².

Treatment with Mitomycin C

The cells were washed with PBS and incubated, in the flask, at 37°C for 2 h with 10 ml /75 cm² flask of DMEM medium-10% FCS containing 4 µg/ml mitomycin C. The mitomycin C was discarded and the cells washed three times with PBS to remove any remaining mitomycin C. If recently passaged the cells were incubated in RM-medium and keratinocytes added or, if confluent the 3T3 cells were trypsinised, counted and plated out at 5×10^5 cells /25 cm² flask in RM-medium.

Surplus treated cells were either stored at 4°C for up to 3 days or were frozen in DMEM medium-10% FCS with 20% DMSO. Frozen cells were recovered in the same way as for untreated 3T3 cells.

3.3 Mycoplasma Testing of Cultured Cells

Infection of cell cultures with mycoplasmas (0.2-0.3 µm) cannot be detected by the naked eye except by deterioration in the culture (Berns, 1983). A common and quick method of detecting mycoplasma involves the use of fluorescent dyes such as Hoechst and DAPI that bind specifically to DNA. Mycoplasma free cultures only show nuclear fluorescence whereas infected cultures show extranuclear fluorescence. Cell cultures were examined for mycoplasma using one of two fluorescent dyes.

Hoechst Stain

The cells were grown on coverslips to 50-80% confluence, fixed in 1 part glacial acetic acid to 3 parts methanol for 15 min and stained with Hoechst stain H33258 (0.05 µg/ml) for 10-30 min at room temperature. The slides were mounted and examined under a fluorescent microscope.

DAPI Stain

The cells were grown on coverslips to 50-80% confluence, stained with DAPI (1 µg/ml in methanol) for 15 min at 37°C, mounted in glycerol and examined under a fluorescent microscope with 340/380 nm excitation filter and LP 430 nm barrier filter. The DAPI stain is taken up into cellular DNA.

Mycoplasma were eliminated by treating the infected cultures with the antibiotic combination BM-Cyclin. This treatment involved 3 or more cycles. For each cycle the cells were grown in BM Cyclin 1 for 3 days followed by 4 days in BM Cyclin 2 containing medium.

3.4 Immunocytochemical Staining of Keratinocytes

Some keratinocyte cultures were grown on glass chamber slides for staining with anti-keratin antibodies (Rugg, 1994). The cells were fixed in acetone/methanol (1:1) for 5 min, air dried and incubated with a drop of the primary antibody for 60 min at room temperature in a humid chamber. The primary antibodies were diluted in tissue culture medium containing 10% FCS (for antibodies used see Appendix III). The slides were rinsed in running water for 5 min, drained and incubated with the secondary antibody (sheep-anti-mouse fluorescein isothiocyanate conjugated, diluted 1:50) for 60 min in a humid chamber. The cells were washed as before, drained and mounted in gelvatol mounting medium. They were examined under a fluorescent microscope for keratin expression and filament network formation.

DNA Analysis

For recipes of buffers, PCR reactions, primers and suppliers of chemicals see Appendix II.

3.5 Extraction of mRNA from Cultured Keratinocytes

Polyadenylated mRNA was isolated directly from cultured keratinocytes or extracted from frozen aliquots of cultured keratinocytes using a Quick Prep Micro mRNA purification kit. The cultured cells were washed 3 times, in the flask, with warm serum-free DMEM medium, scraped off into a microfuge tube and centrifuged for 30 sec. Frozen keratinocytes were thawed and washed 3 times in warm serum free DMEM medium, centrifuging at 1,000 rpm for 20 sec to pellet the cells between each wash. The cells were then extracted in a buffer containing a high concentration of guanidinium thiocyanate and N-lauroyl sarcosine, to inactivate endogenous RNases. The extract was diluted in 10 mM Tris-HCl (pH 7.5), 1mM EDTA to a level that still inhibited RNases but was low enough to allow hydrogen bonding between polyadenylated tracts on the mRNA molecules and oligo (dT) attached to cellulose. The extract was centrifuged and the supernatant transferred to a microfuge tube containing Oligo (dT)- Cellulose. The samples were mixed for 3 min to allow the polyadenylated RNA to bind to the Oligo (dT)- Cellulose, centrifuged, and the supernatant discarded.

The pelleted material was then washed five times in a high-salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl), and twice in a low-salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl) with a 10 sec centrifugation between each wash. The pellets were suspended in low-salt buffer and transferred to a Microspin Column within a microfuge tube and the column washed three times with low-salt extraction buffer. The polyadenylated material was then washed twice in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. Each 400 µl sample was then divided into 2 x 200 µl aliquots and precipitated with 5 µl glycogen solution, 20 µl potassium acetate solution and 500 µl

ethanol. The mRNA pellets were either stored at -70°C in 100% ethanol or redissolved in 50 μl DEPC water and an aliquot used for reverse transcription (5 μl used per 20 μl reverse transcription reaction). Any remaining mRNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volume 100% ethanol and stored at -70°C .

mRNA stored in 100% ethanol was recovered by centrifuging for 15 min at 14,000 rpm at 4°C . The supernatant was discarded, the pellet washed with 70% ethanol and recentrifuged for 15 min at 14,000 rpm. Any excess ethanol was allowed to evaporate from open tubes or by speed vacuum drying.

3.6 Reverse Transcription of mRNA to cDNA

The mRNA was reverse transcribed to produce cDNA using primers specific to either keratin 5 or keratin 14. To avoid contamination by RNases all tubes were autoclaved and solutions were made up in DEPC water. To 5 μl of RNA (or water as a control) 1 μl of a 21 base oligonucleotide primer (to either keratin 5 or keratin 14; 0.5 mg/ml), and 0.5 μl RNasin (20 units) was added. After mixing, the reactions were incubated at 70°C for 10 min and cooled to 37°C . The incubation was continued in the presence of 1 μl of PCR buffer x 20 concentration (0 mM MgCl_2), 1 mM dithiothreitol (DTT), 7mM MgCl_2 , 1 mM 2' deoxynucleoside 5' triphosphate (dNTP) and 5 units of AMV reverse transcriptase. The final reaction volume was made up to 20 μl with diethyl pyrocarbonate (DEPC) treated water and the reactions were incubated at 37°C for 60 min. After cooling to room temperature, the cDNA was used as a template for PCR reactions or, stored at -20°C until required.

3.7 Isolation of Genomic DNA from Whole Blood

Genomic DNA was extracted from whole blood obtained from both affected and unaffected members of a family. The DNA was PCR amplified to produce keratin 5 and keratin 14 DNA for sequencing and genetic linkage analysis.

Whole blood (20 ml) was poured into a 50 ml centrifuge tube topped up with ice cold double distilled water and left on ice for 30 min to lyse the erythrocytes. (If the blood was partially clotted it was first homogenised). After centrifuging at 6,000 rpm for 10-15 min at 4°C the pellet was resuspended in 25 ml of ice cold triton lysis mix pH 7.7 and left on ice for a further 20 min. The cells were centrifuged at 6,000 rpm for 10 min at 4°C and the pellet resuspended in 9 ml of NaCl/EDTA (0.75 ml NaCl/0.025 M EDTA pH 8.0). The DNA was released from the nucleus by the addition of 500 µl of 10% sodium dodecyl sulphate (SDS) and 200 µl proteinase K (10 mg/ml) which lysed the white cell membranes and the nuclear membranes. The DNA was incubated either at 55°C for 3 h or at 37°C overnight.

If the preparation was clean (colourless), 1/3 volume of 6 M NaCl was added, shaken vigorously for 20 sec and centrifuged at 6,000 rpm at 4°C for 15 min. The NaCl precipitated all white cellular membranes and organelles, leaving the DNA suspended in the supernatant. If the sample was dirty, 10 ml of phenol : chloroform (1:1) was added and the preparation centrifuged for 10 min at 6,000 rpm. The supernatant was removed, 1/3 volume of 6 M NaCl added, shaken vigorously for 20 sec and centrifuged at 6,000 rpm at 4°C for 15 min. The supernatant, from either preparation, was carefully poured into a clean tube, 2 volumes of 100% ethanol added and the tube swirled to precipitate the DNA out as a white clump. The DNA was left at room temperature or at -20°C for 1 h and then carefully hooked out into a microfuge tube. The DNA was washed in 1 ml of 70% ethanol, centrifuged for 1 min, dried and

dissolved in 450 µl of sterile Tris-EDTA (TE) buffer. The optical density of the DNA was read at 260 nm and 280 nm and the concentration of the DNA calculated.

3.8 Polymerase Chain Reaction (PCR)

PCR is a technique to amplify exponentially a specific fragment of DNA and produce many copies of it. A DNA polymerase, template, primer and deoxyribonucleotide triphosphates are required to synthesise a new strand of DNA complementary to the template. For each cycle the double stranded DNA is separated by heating to a high temperature (94°C), the primers are annealed to the complementary piece of target DNA and the DNA is extended using the DNA polymerase. The use of thermostable polymerases e.g. *Thermophilus aquaticus* (Taq), which can survive high denaturing temperatures (96°C), avoids having to add fresh enzyme after each cycle (Saiki, 1989).

cDNA from the cultured keratinocytes was amplified by PCR using the same oligonucleotide primer as for the reverse transcription reaction and a second primer, to produce a product of 1427 base pairs for keratin 5 and 1325 base pairs for keratin 14. Keratins K5 and K14 were amplified from the genomic DNA using several sets of overlapping primers. PCR is a very sensitive technique and contamination with a single target molecule can lead to false positive signals, therefore it was important to ensure that all solutions were free from contaminating DNA and that a water control was included for each set of reactions. Thirty cycles were sufficient to produce enough DNA for further studies. The PCR reactions (100 µl total volume) were incubated in a thermal cycler, under the following conditions: 1 cycle of 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 1 min at 60°C and 2 min at 72°C and a final cycle of 5 min at 72°C. PCR amplified DNA (5 µl) was resolved on a 1.5% agarose gel to check the purity, size and yield of DNA. The DNA was stored at -20°C until required.

Detection of PCR Products by Agarose Gel Electrophoresis

Agarose gels of differing concentrations were used to separate DNA fragments ranging in size from about 150 base pairs to 1,500 base pairs. The DNA was stained with ethidium bromide, a fluorescent dye which binds to the DNA. The ethidium bromide was included in the agarose gel allowing the DNA to be visualised under ultraviolet light as soon as the gel was resolved.

The PCR products (5-10 µl) were resolved on agarose gels (usually 1.5%) with standard DNA VI molecular weight markers (2176, 1766, 1239, 1033, 653, 517, 453, 394, 298, 234, 220 and 154 base pairs) run alongside to estimate the size and yield of the DNA. Small electrophoresis kits were used requiring 50 ml of agarose gel and 50 ml of running buffer. The agarose was dissolved in either Tris-acetate EDTA (TAE) buffer (1 x concentration), or in Tris-borate EDTA (TBE) buffer (0.5 x concentration), using a microwave (2-3 min on medium power). The agarose was cooled to approximately 60°C, ethidium bromide added (final concentration 0.5 µg/ml) and the agarose poured into a tank containing the appropriate size comb and allowed to set. DNA samples were mixed in the appropriate sample buffer, TAE/TBE buffer x 6 concentration. The running buffer was poured into the tank, the samples loaded and electrophoresis carried out at 1-5 V/cm.

3.9 Purification of Oligonucleotide Primers

Oligonucleotides of between 18-22 base pairs were chosen from the published sequences of keratin 5 (Eckert & Rorke, 1988; Lersch & Fuchs, 1988) and keratin 14 (Hanukoglu & Fuchs, 1982; Marchuk *et al.*, 1984, 1985) and chemically synthesised for use as primers to amplify specific fragments of DNA by PCR and for DNA sequencing. The oligonucleotides were purified by one of two methods, depending on whether they were synthesised with the dimethoxytrityl (DMT) group still attached at the 5' terminus.

Purification of Oligonucleotides with the DMT Group Attached by Reverse Phase High Performance Liquid Chromatography

Each unpurified oligonucleotide (1.5 ml) was purified on a Poly-Pak cartridge. Full length oligonucleotides containing the DMT group attached to the polymeric resin while failure sequences did not. The DMT group was removed by flushing the cartridge with 2% tri-fluoroacetic acid (TFA) and the purified oligonucleotide was eluted in 20% acetonitrile, dried in a speed vacuum and resuspended in DEPC water. The concentration was calculated from the optical density at 260 nm ($A_{260} = 1 = 80 \mu\text{g/ml}$ of single stranded DNA) and the purified oligonucleotide diluted appropriately.

Purification of Oligonucleotides Without the DMT Group Attached by Precipitation with Sodium Acetate and Ethanol

To 300 μl of unpurified oligonucleotide, 30 μl of 3 M sodium acetate (pH 5.8) and 750 μl of 100% ethanol was added. After mixing they were left on ice for 30 min, then centrifuged at 14,000 rpm for 10 min. The pellet was washed in 70% ethanol, re-centrifuged at 14,000 rpm for 10 min, dried in a speed vacuum and redissolved in 300 μl DEPC water. The optical density was read at 260 nm and the purified oligonucleotide diluted to the appropriate concentration for use.

3.10 Purification of PCR Amplified DNA

The PCR amplified cDNA and genomic DNA were purified prior to direct cycle sequencing to remove excess nucleotides and amplification primers that could otherwise interfere with the sequencing reactions by producing false stops. The DNA was purified either by differential precipitation with isopropanol (Brow, 1990), or by low melting point gel electrophoresis. Low melting point gel agarose has hydroxyethyl groups introduced into the polysaccharide chain, allowing the agarose to gel at about 30°C and to melt at about 60°C, well below the melting temperature of most double stranded DNA, thereby allowing the DNA to be recovered (Sambrook *et al.*, 1989).

Purification of DNA by Isopropanol Precipitation

Approximately 400 µg of PCR amplified DNA (estimated by resolving a small sample on an agarose gel) was mixed with 1 volume of DEPC water, 2 volumes of 4 M ammonium acetate and 4 volumes of isopropanol. Each sample was left on ice for 10 min to precipitate out the DNA and centrifuged at 14,000 rpm for 10 min. The supernatant was discarded and the pellet washed with 70% ethanol, re-centrifuged at 14,000 rpm and the DNA pellet allowed to dry either in the speed vacuum (approximately 10-15 min) or in an open tube on the bench overnight. The purified DNA was redissolved in DEPC water to a concentration of 5 ng/µl.

Purification of DNA by Low Melting Point Gel Electrophoresis

Low melting point agarose gels (1.5%), containing 0.5 µg/ml ethidium bromide, were prepared in TAE buffer (x1 concentration) using combs of the appropriate size for the amount of DNA to be purified. After cooling to room temperature, the gels were placed at 4°C to allow complete setting. The TAE running buffer (x1 concentration) was also cooled to 4°C. The PCR amplified DNA was mixed with TAE sample buffer (x 6 concentration), loaded onto the gel and electrophoresis carried out at 4°C at 1-5 V/cm. DNA molecular weight standards were run alongside the samples. The DNA was visualised with a UV light for the minimum amount of time, the appropriate band carefully cut out and placed in a pre-weighed microfuge tube. The tube was reweighed to calculate the approximate volume of gel (assuming a density of 1 gm/ml). To the DNA 0.2 volume of 3 M sodium acetate (pH 5.5) and 0.1 volume of 1 M Tris-HCl (pH 8.0) was added and the mixture heated to 70°C to dissolve the agarose. One volume of phenol was added and after vortexing for 30 sec the mixture was placed on dry ice/ethanol until frozen and centrifuged at 14,000 rpm for 10 min at room temperature. The aqueous layer was removed and precipitated with 2.5 volumes of 100% ethanol for 30 min at 4°C. The purified DNA was pelleted by centrifuging at 14,000 rpm for 10

min at room temperature and dried in the speed vacuum or in an open tube on the bench. The DNA was redissolved in DEPC water to give 5 ng/ μ l.

The yield of the purified DNA can be checked by resolving a small aliquot of the DNA on a 1.5% agarose gel but this was not routinely carried out.

3.11 Double Stranded DNA Cycle Sequencing

Purified keratin 5 and keratin 14 cDNA and genomic DNA was sequenced directly using a double stranded (ds) DNA cycle sequencing protocol. The method was based on Sanger's dideoxy chain termination procedure (Sanger *et al.*, 1977). DNA polymerase catalysed the reaction between a labelled oligonucleotide that was complementary to the DNA being sequenced. The double stranded DNA was added to a set of four nucleotide specific sequencing reactions (adenine, A; cytosine, C; guanine, G or thymine, T). By a series of temperature changes (carried out in a thermal cycling machine) the DNA was heat denatured, the end labelled primer annealed and the primers then extended as complementary oligonucleotides until the addition of a base specific dideoxyribonucleotide (A, C, G, or T) which terminated the reaction. End-labelling the primers with γ 32 P ATP had the advantage that only the sequence from the end-labelled primer was detected, therefore the sequence could be read starting several bases after the 3' end of the primer.

Approximately 25 ng (5 μ l) of purified DNA was used per 36 μ l reaction volume (for primers and reaction mixes see Appendix II). The cycling parameters for the sequencing reactions were 20 cycles of 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C followed by 10 cycles of 30 sec at 94°C and 1 min at 72°C. The synthesis products from the reaction (5 μ l) were separated by length on a 6% polyacrylamide/TBE-urea gel. The gels were preheated to 50°C before the samples were loaded and then run at this temperature for 2-4 h. They were then dried for 1 h

and exposed to X-ray film for 24-48 h. The sequences obtained were compared with those published for normal keratin 5 (Eckert & Rorke, 1988; Lersch & Fuchs, 1988) and keratin 14 (Hanukoglu & Fuchs, 1982; Marchuk *et al.*, 1984, 1985).

Approximately 150-200 base pairs could be read from one reaction; overlapping primers were used to obtain the full coding sequences of keratin 5 and 14. A control DNA sample, either from TR146 cells or from an unaffected individual, was included for each reaction.

3.12 Exclusion of Polymorphisms

Genomic DNA from affected and unaffected members of the family and from 50 unrelated controls was screened to exclude the possibility that a keratin gene mutation discovered was an innocuous polymorphism occurring within the normal population. The method chosen for this depended on the base change. If the mutation created a new restriction enzyme site, this was used to screen samples. A fragment of DNA was amplified by PCR, digested with the appropriate enzyme, and the digests resolved on an agarose gel. Those samples with the mutation and therefore a new enzyme site produced an additional fragment. If the mutation did not create a restriction enzyme site, a primer was designed which, in conjunction with the identified mutation, created a new restriction enzyme site. DNA was amplified with this primer, digested with the enzyme and the samples resolved on an agarose gel. An additional fragment was observed in samples containing the mutation. Alternatively PASA analysis (PCR amplification of specific alleles) method was used (Sommer *et al.*, 1992) where an exact match of the 3' base of the primer was required for amplification by PCR. A primer specific to the mutation, with the mutant base at the 3' position of the primer, only amplified samples containing the mutation. With the normal primer all samples were amplified.

Family A

Screen for the Mutation in the Linker L12 of Keratin 5 (Arg331-Cys)

The mutation identified in the L12 linker of keratin 5 did not create or destroy a restriction enzyme site therefore, a primer was designed, which in conjunction with the identified mutation, created a new *AluI* restriction enzyme site. After PCR amplification with this primer, the DNA was digested with the enzyme *AluI*. Only samples with the mutation cut at this new site resulting in an additional fragment when the digests were resolved on a sequencing gel and visualised by autoradiography.

PCR reactions (15 µl total volume) were set up in the presence of $\alpha^{32}\text{P}$ ATP and incubated for 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 1 min at 60°C, 2 min at 72°C and 1 cycle of 5 min at 72°C. To 15 µl PCR product, 1.5µl of enzyme buffer (x 10 concentration) and 5 mM spermidine was added. The samples were mixed and incubated for 10 min at 65°C, cooled to room temperature and then incubated with *AluI* (1 U/15 µl) overnight at 37°C. Formamide-EDTA buffer was added (8 µl/15 µl), the samples incubated for 5 min at 90°C and resolved on a 4% polyacrylamide TBE-urea sequencing gel. The gels were dried for 1 h and exposed to X ray film for 24-48 h.

Family B

Screen for the Mutation in the L12 Linker of Keratin 14 (Val270-Met)

The screen for this mutation was based on the principle that an exact match of the 3' base of the primer was necessary for PCR amplification of the DNA (PASA analysis). A 130 base pair fragment was amplified by PCR. Only samples with the mutant allele were amplified with the primer specific to the mutation, while all samples were amplified when the normal wild type primer was used.

PCR reactions (25 µl final volume) were incubated for 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 45 sec at 65°C and 1 min at 72°C followed by 5 min at 72°C. PCR samples were resolved on a 1.5% agarose gel.

Family C

Screen for the Mutation in the 1A Domain of Keratin 5 (Asn193-Lys)

This mutation created a new *MboII* restriction enzyme site. A 288 base pair fragment of genomic DNA was amplified by PCR and digested with *MboII* to produce two fragments in normal keratin 5 of 52 and 236 bases pairs. Samples containing the mutation and therefore an additional site produced four fragments after digestion of the amplified fragment with *MboII* (52, 64, 172 and 236 base pairs).

The cycling parameters for the PCR reactions (50 µl total volume) were 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 45 sec at 60°C and 1 min at 72°C followed by 5 min at 72°C. PCR samples (5 µl) were resolved on a 2% agarose gel (TBE buffer) to check amplification before digestion with *MboII*. *MboII* enzyme (1 U) and 4.5 µl of the enzyme buffer (x 10 concentration) was added to the remaining 45 µl PCR amplified DNA and the samples were incubated at 37°C overnight. Samples (20 µl) were resolved on a 4.5% Nusieve agarose gel (TBE buffer).

Family D

Screen for Mutation in the 2B Domain of Keratin 14 2B (Arg388-Cys)

Several approaches to screen for this mutation were carried out, including PASA analysis and designing a primer to create, in conjunction with the mutation, a new *PstI* restriction enzyme site. The high sequence homology of keratin 14 with a keratin 14 pseudogene caused problems in screening genomic DNA for this mutation. Given below is one method that appeared to overcome this, although insufficient samples

were screened to statistically confirm that this mutation causes EBS and is not a common polymorphism (see Chapter 4, 4.2).

A fragment of DNA was amplified by PCR (50 µl volumes) under the following conditions; 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 1 min at 60°C and 1 min at 72°C followed by 5 min at 72°C. The PCR products were isopropanol precipitated, and then re-amplified with two internal primers, one of which created the new *Pst*I site. The PCR reactions (50 µl final volume) were incubated for 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 45 sec at 64°C and 1 min at 72°C followed by 5 min at 72°C. *Pst*I enzyme (2U) and 5 µl enzyme buffer (10 x concentration) were added to the amplified DNA (50 µl) and the samples incubated at 37°C overnight. Digests were resolved on a 4% Nusieve agarose gel.

3.13 Genetic Linkage Analysis

Genetic linkage analysis, using naturally occurring DNA polymorphisms, was used to predict which chromosome carried the defective allele responsible for the EBS phenotype in a particular family. Polymorphisms, occur naturally within the human genome either as single nucleotide substitutions or involving several base pairs to produce DNA insertions or deletions (complex polymorphisms); most have no effect on the function of the gene. Alteration of a single nucleotide may create or delete a restriction enzyme site which can be detected by PCR amplification of the DNA fragment followed by digestion with the appropriate enzyme and gel electrophoresis. Complex polymorphisms can exist in the form of variable number of tandem repeats (VNTR) where differences in the number of repeats of short sequences can lead to multi-allelic polymorphisms. VNTRs can be detected by PCR amplification of the repeat sequence followed by gel electrophoresis to visualize the DNA fragments of variable sizes.

Linkage analysis was performed on genomic DNA from affected and unaffected members of the families. Polymorphic markers within or near the keratin gene clusters on chromosome 12 (type II) and chromosome 17 (type I) were used to determine whether the disease mapped to either type I or type II keratins and included; *AvaII* restriction fragment length polymorphism (RFLP) of human keratin 10 (McLean & Lane, 1992) D17S800 and *BsaII* and *MaeII* RFLP of human keratin 8 (McLean, personal communication). Some linkage data was also obtained by sequencing fragments of DNA containing known polymorphisms; K14, codon 77, 94, and 123 (Coulombe *et al.*, 1991a); K5, codon 138, 197 and 198 (Chan *et al.*, 1993) from several members of a family (both affected and unaffected). Co-segregation of a DNA polymorphic marker with the disease in every affected member of a family indicated that the defective allele was on the same chromosome as the DNA marker, i.e. chromosome 12 or 17, making it likely that either the keratin 5 or keratin 14 gene respectively was abnormal. The data was analysed using the MLINK programme.

***AvaII* Restriction Fragment Length Polymorphism (RFLP) of Human Keratin 10**

Intron III of the keratin 10 gene was amplified by PCR using primers in the flanking exons. Digestion of the 769 base pair product with *AvaII* restriction enzyme produced fragments of 647 and 122 base pairs (McLean & Lane, 1992).

The PCR reactions (25 µl final volume) were incubated under the following conditions: 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C and 1 cycle of 5 min at 72°C. PCR samples (5 µl) were resolved on 1.5% agarose gels (TBE buffer) to check amplification. The remaining 20 µl was digested with *AvaII*; 2 µl of enzyme buffer (x 10 concentration) and 0.2 µl (0.8 U) *AvaII* enzyme were added and incubated overnight at 37°C. Digested samples (15 µl) were resolved on a 1.5% agarose gel (TBE buffer).

D17S800

This CA repeat polymorphism maps to chromosome 17. A 160 base pair fragment was amplified by PCR in the presence of one primer end labelled with $\gamma^{32}\text{P}$ ATP. The reactions (25 μl final volumes) were incubated for 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C. The PCR products were resolved on a polyacrylamide/TBE-urea sequencing gel. The gel was dried for 1 h and exposed to X-ray film for 24-48 h.

***Bsa*II and *Mae*II Restriction Length Polymorphism (RFLP) of Human Keratin 8**

A 350 base pair fragment from the keratin 8 gene was PCR amplified. Digestion of the fragment with *Bsa*II restriction enzyme produced fragments of 70 and 280 base pairs, and digestion of the 350 base pair fragment with the restriction enzyme *Mae*II produced fragments of 72 and 278 base pairs (personal communication W.H.I.McLean).

The cycling parameters for the PCR reactions (100 μl final volume) were 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C. The PCR products (5 μl) were resolved on 1.5% agarose gels (TBE buffer) to check amplification. The remaining 95 μl was divided into two aliquots (40 μl) and digested with either *Bsa*II or *Mae*II. To each aliquot, 4 μl of enzyme buffer (10 x concentration) and 1 U of the appropriate enzyme was added. Those with the *Bsa*II enzyme were incubated at 60°C overnight and those with *Mae*II enzyme at 50°C overnight. The digested samples (20 μl) were run out on 4% Nusieve agarose gels (TBE buffer).

Sequencing Data of Polymorphisms

DNA fragments containing known polymorphisms were sequenced from genomic DNA from affected and unaffected members of a family.

The following polymorphisms were examined:

Keratin 14

codon		base	amino acid
77	231 C-T	AGC-AGT	Ser-Ser
94	280 G-A	GCT-ACT	Ala-Thr
123	369 T-C	AAT-AAC	Asn-Asn

Keratin 5

codon		base	amino acid
138	804 G-A	GGA-GAA	Gly-Glu
197	982 C-A	GAC-GAA	Asp-Glu
198	985 C-A	ACC-ACA	Thr-Thr

Protein Analysis

For recipes of buffers, gels and antibodies used see Appendix III.

3.14 Extraction of Cytokeratins from Keratinocyte Cultures

Cytokeratins were extracted from keratinocytes cultured from EBS patients, unaffected, unrelated controls and from TR146 cells (Stasiak *et al.*, 1989). The tissue culture medium was discarded and the cells, in a 25 cm² flask, were washed with 3 x 10 ml of warm PBS or serum free DMEM medium. The following stages were carried out at 4°C to reduce protein degradation. Low salt extraction buffer (3-5 ml) was added to the cells and they were then left on ice for 20 min. The low salt extraction buffer was replaced with 3-5 ml of high salt extraction buffer and the cells incubated on ice for a further 20 min. The cells were washed 3 times with 10 ml of wash buffer, scraped off the flask and transferred to a centrifuge tube. The cells were pelleted by centrifuging at 2,000 rpm for 15 min and resuspended in 1 ml of sodium dodecyl sulphate (SDS) sample buffer. The extracts were aliquoted into 1.5 ml Eppendorf tubes and boiled for 5 min before loading onto a gel or storing at -70°C.

3.15 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The cytokeratins were solubilised with the denaturant sodium dodecyl sulphate (SDS) and resolved on one dimensional discontinuous SDS PAGE slab gels (Laemmli, 1970) in the presence of a reducing agent, dithiothreitol. Polyacrylamide gels were formed by polymerisation of acrylamide monomers into long chains that were cross-linked with N N' methylene bisacrylamide. The rate of migration of a protein through an acrylamide gel was proportional to the pore size which is determined by the concentration of acrylamide and degree of cross-linking; as the acrylamide concentration increases the pore size decreases. Samples were loaded onto a large pore stacking gel where, during migration, the proteins were concentrated into very narrow bands before being separated according to size and charge, in the smaller pore resolving gel.

The keratins were resolved on 10% acrylamide gels with 2% cross linking and were stained with Coomassie blue stain (which stains all proteins) or transferred onto nitrocellulose paper by Western blotting for immunostaining (Rugg, 1994). Routinely mini gels (8 cm x 8 cm x 0.75 cm) were run, larger gels (12 cm x 15 cm x 1 cm) were used when higher resolution was required.

The glass plates were cleaned thoroughly with distilled water and methanol, to remove any traces of dust in the air that is derived from skin and could cause contamination, and assembled in the cassettes according to the manufacturers instructions. For 5 mini gels 30 ml of resolving gel was sufficient or 20 ml for one large gel. The N, N, N', N'- tetramethylethylenediamine (TEMED) and 10% ammonium persulphate were added last to the gel mixture as the TEMED initiates polymerization of the acrylamide by catalysing free radicals from the ammonium persulphate. The resolving gels were poured and overlaid with 80% isopropanol to give a level gel surface and to exclude oxygen which inhibits polymerisation of the acrylamide. After 30-60 min the isopropanol was removed from the polymerised resolving gels and the surface of the gels were rinsed with distilled water to remove any unpolymerised gel. Just before use, 2 ml stacking gel per mini gel or 10 ml per large gel was added and allowed to set for about 15 min. The stacking gel should be twice the height of the sample volume. The comb was removed, the wells rinsed with electrode buffer to remove any unpolymerised gel, and the upper and lower reservoirs were filled with electrode buffer.

Sample Loading and Running of Gels

The keratin samples were boiled for 5 min and centrifuged at 12,000 rpm for 3 min to precipitate any undissolved material. Samples (10 µl) were loaded onto each lane of a mini gel and unused wells were filled with the equivalent volume of SDS sample buffer. Prestained molecular weight markers (low range 18.5-106 kD; phosphorylase

B = 106 kD; bovine serum albumin = 80 kD; ovalbumin = 49.5 kD; carbonic anhydrase = 32.5 kD) were run together with cytoskeletal extracts from TR146 cells (known to express keratins K5, K6, K14, K17, with smaller amounts of keratins K8, K18 and K19; Rupniak *et al.*, 1985) to act as markers and controls. The prestained molecular weight markers run at different molecular weights than the original proteins due to the covalently attached dye. The gels were run at 25-30 mA for 1.5-2 h until the bromophenol blue dye front reached the bottom of the gel. Extra resolving gels were stored for up to a week at 4°C wrapped in wet paper towels and saran wrap.

Coomassie Blue Staining

Gels were incubated in Coomassie blue stain for 2-12 h at room temperature, shaking gently. The stain was decanted off and replaced with destain solution and the gels were left shaking at room temperature. The destain solution was replaced several times until most of the background staining had been removed.

3.16 Western Blotting

The separated proteins were transferred from the SDS gels by Western blotting (i.e, by electrophoretic transfer) onto a nitrocellulose membrane to which they were immobilised and tightly bound. The proteins were transferred onto 0.22 µm nitrocellulose paper by assembling a 'sandwich' of the gel and the nitrocellulose membrane between a porous supporting material (3 MM Whatman filter paper and Scotchbrite sponge) and immersing it in the transfer buffer in an electroblotter tank. Transfer was carried out at 180 mv for 1 h.

3.17 Staining of Western Blots with Anti-Keratin Antibodies

Keratins bound to the nitrocellulose membrane were immunologically detected by reacting the blots with monoclonal antibodies to the keratins of interest. Detection of the primary antibody depends on the type of secondary antibody used. Here, the

secondary antibody was conjugated to alkaline phosphatase which converts the substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP) into a compound which reacts with nitro blue tetrazolium (NBT) to produce an insoluble dark blue colour visualised as band(s) corresponding to the protein.

The blots were blocked overnight in 0.05% TWEEN-20 in PBS (20% Marvel milk powder for 1 h can be used but the blots cannot then be stained for total protein) to prevent non specific binding of the primary or secondary antibodies. All incubations were carried out at room temperature on a rotary shaker. After washing for 5 min in tap water the blots were incubated with the diluted primary antibody for 1 h (5 ml for 8 cm x 8 cm blot), washed with tap water for 5 min and then incubated with the secondary antibody (rabbit anti-mouse alkaline phosphatase conjugated antibody) for 1 h. The blots were washed in tap water as before for 5 min and incubated in wash buffer for 15 min. After briefly washing in tap water, the blots were incubated in fresh substrate solution (containing NBT and BCIP) for 2-10 min, shaking gently until the dark blue/purple colour developed. The reaction was stopped by washing the blots briefly in tap water before air drying them on blotting paper.

The specificity of reaction was tested by incubating a strip of a blot with secondary antibody alone.

3.18 Staining of Western Blots for Total Protein

The blots were stained for total protein using Ponceau S stain and India Ink.

Ponceau S Staining

Ponceau S is a reversible protein dye which was used to check the transfer of protein from the gel onto the nitrocellulose paper after Western blotting. The protein is stained

red but the dye can be washed from the blot and does not interfere with further reactions.

The blots were rinsed briefly in distilled water, incubated in Ponceau S for 5 min while shaking and rinsed again in distilled water. After visualising the protein bands, the blots were totally destained by further washing and then stained with monoclonal antibodies.

India Ink Staining

India ink stains all proteins and can be used on blots already stained with monoclonal antibodies. The blots were incubated in 0.3% TWEEN-20 in PBS for at least 15 min, washed briefly in tap water and incubated in India ink at room temperature on a rotary shaker. When sufficiently stained (0.5-18 h), the blots were washed with tap water and air dried on blotting paper.

CHAPTER 4

KERATIN STUDIES: RESULTS

4.1 Keratinocyte Cell Culture

Skin biopsies were obtained for keratinocyte culture from clinically normal skin, from at least one member of each of four EBS-WC families studied. When the project was initially started the cells were to be used for both enzyme and keratin studies.

However, as the project progressed and attention was concentrated on keratin studies, cytokeratins and mRNA were extracted from these cells (see Chapter 3).

The keratinocytes were cultured on a monolayer of Swiss mouse 3T3 feeder cells and attached to the culture dish as single cells or small clusters. From these, keratinocytes grew out from the periphery of the colonies, pushing aside the 3T3 feeder cells to form a continuous sheet about 8 days after seeding. This consisted of a multilayered sheet of cells and although there was no stratum corneum the cells underwent terminal differentiation. If not subcultured the keratinocytes eventually detached from the flask.

The keratinocytes cultured from skin biopsies from patients with EBS did not show any abnormal growth characteristics compared with keratinocytes cultured from human foreskin. They did not show any tendency to blister as has been reported. Leigh *et al.* (1984), demonstrated that keratinocytes cultured from patients with the three main types of EB, simplex, junctional and dystrophic, formed blisters after 14-22 days in culture, before confluence was reached. Although, this abnormal blistering was most marked in keratinocytes from junctional EB patients, blister formation was reported in the one EB simplex culture examined, after 20 days in culture. Control cultures did not show any blistering even at confluence. The majority of my cultures though, were subcultured before 14 days which would be one explanation why no blistering was observed.

Figure 10a-d shows a series of photographs of subcultured keratinocytes from an EBS affected member of family A. One day after plating onto mitomycin C treated 3T3 feeder cells small colonies of keratinocytes had attached to the flask and rapidly expanded during the next nine days to produce an almost confluent culture. The 3T3 feeder cells were pushed aside by the expanding keratinocyte colonies and eventually became detached from the flask.

Cultured keratinocytes express keratins K6, K16 and K17 in addition to the basal cell keratins K5 and K14 (Fuchs & Green, 1978; Sun & Green, 1978). These keratins were identified in keratin extracts resolved on SDS polyacrylamide gels, followed by immunoblotting and staining with monoclonal antibodies (see Chapter 4, 4.2).

However, many of the biochemical changes characteristic of terminal differentiation, such as expression of the larger suprabasal keratins, K1 and K10 and of filaggrin, do not occur under normal culture conditions (Fuchs & Green, 1980) even when the calcium level in the medium is increased. Calcium promotes stratification and differentiation in human keratinocytes is induced at a concentration of > 0.1 mM in tissue culture medium to produce the morphological characteristics of suprabasal epidermal cells (Pillai *et al.*, 1990). If the calcium concentration is reduced to 0.03-0.1 mM the uniform polygonal cells still proliferate but do not differentiate or stratify. In low calcium concentrations there is little cell-cell contact as desmosomes, the junctions that hold epidermal cells together, are not formed. The desmosomal proteins are still synthesized, but are located throughout the cytoplasm (Watt *et al.*, 1984). However, increasing the calcium concentrations results in rapid formation of desmosomes, and rearrangement of the keratin filaments towards the cell periphery and, later stratification occurs (Watt *et al.*, 1984). Zamansky *et al.* (1991) report simultaneous changes in all three cytoskeletal networks, microfilaments, keratin intermediate filaments and microtubules, when cultured keratinocytes undergo a calcium switch from low to high in the culture medium.

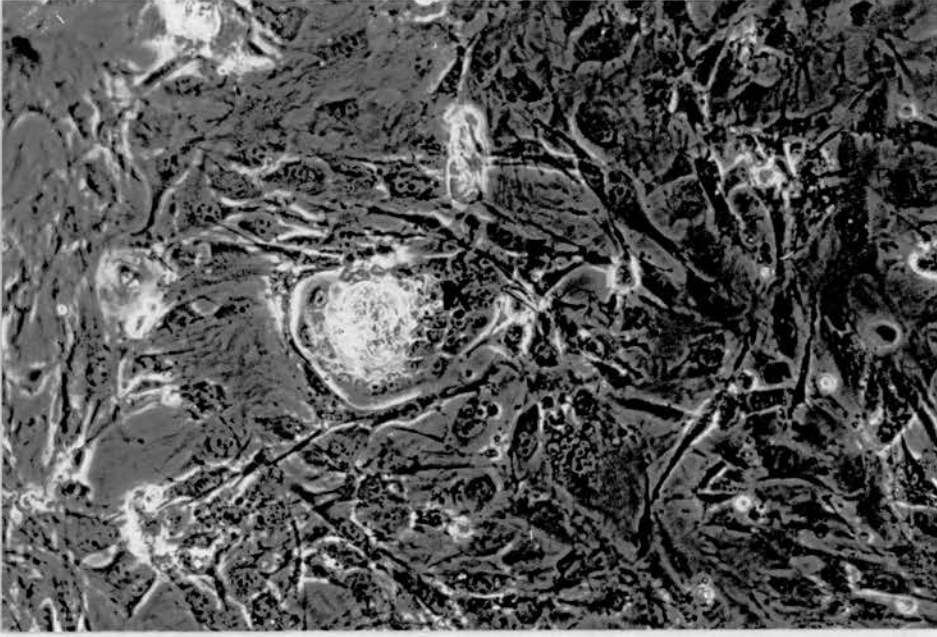


Figure 10a. One day after seeding onto a layer of mitomycin C treated 3T3 feeder cells, small keratinocyte colonies were observed. K marks a keratinocyte colony and F the 3T3 feeder cells.

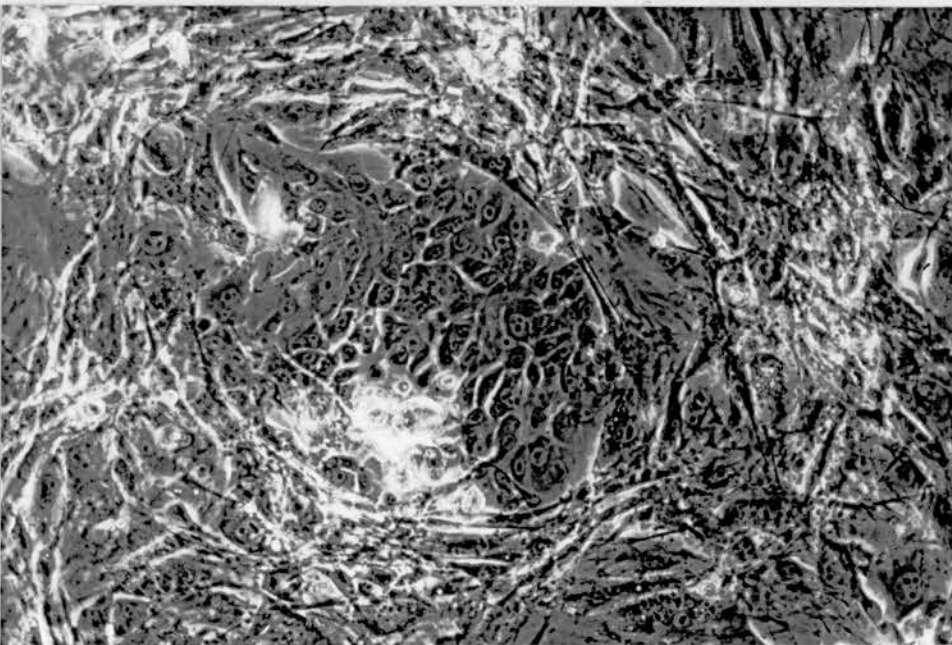


Figure 10b. By day three the keratinocyte colony had expanded pushing aside the feeder cells.

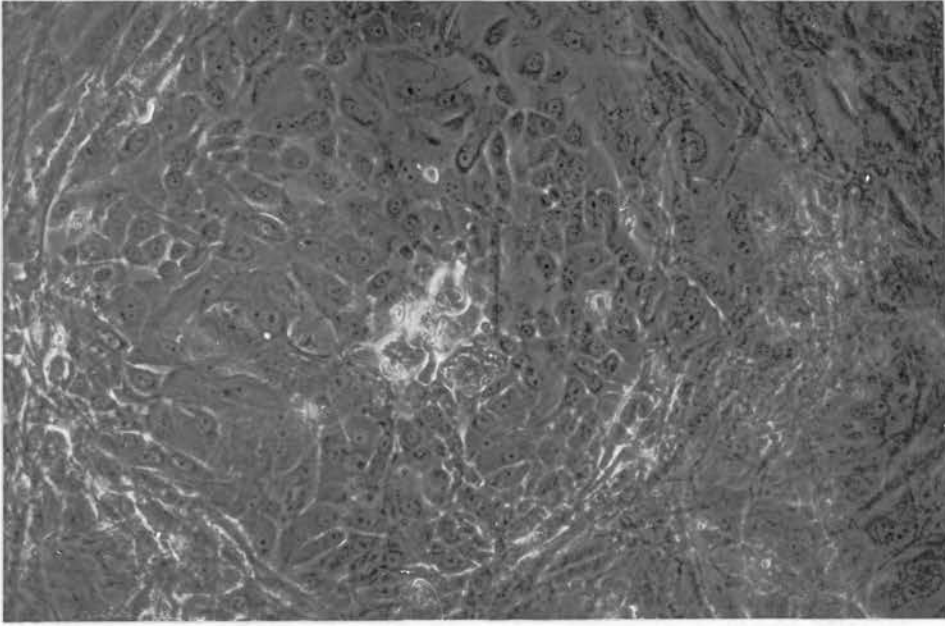


Figure 10c. Day four shows continued growth of the colony.

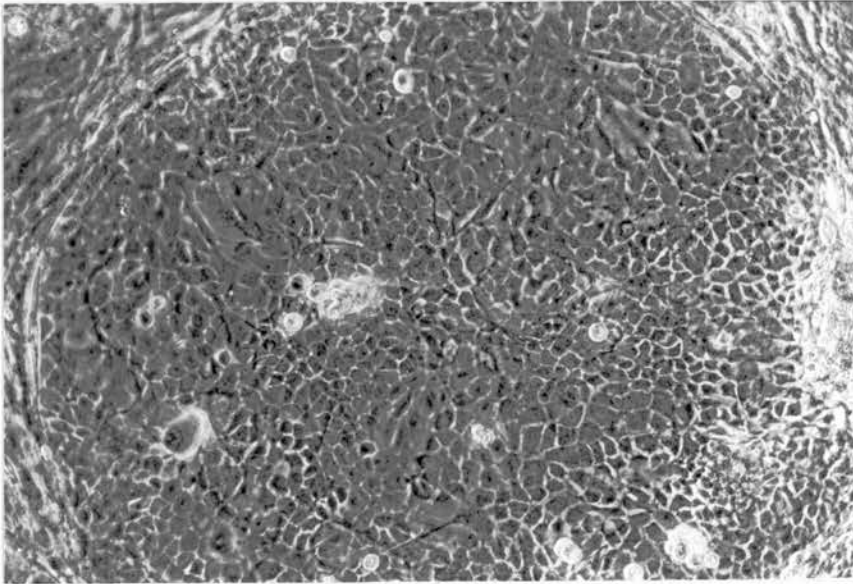


Figure 10d. An almost confluent culture was obtained by day 10 with just the remains of the feeder cells between the colonies.

4.2 Families A and B: Keratin Gene Mutations in the L12 Linker Domain

Point mutations were identified in the central L12 linker domain of two EBS-WC families. In family A, the mutation was in the L12 linker of keratin 5 at codon 331 and resulted in an amino acid change from the predicted arginine to a cysteine residue. In the second, family B, an amino acid change from the predicted valine to methionine was identified at codon 270 in the L12 linker of keratin 14.

4.2.1 Family A

Clinical Description

Detailed clinical information from this family (figure 11) and, from the other four families studied in my project, was recorded by Dr. H. M. Horn and Dr. M. J. Tidman (Dermatology Department, Edinburgh Royal Infirmary) when patients were interviewed for the UK National EB Register (see Appendix IV). The age of onset, distribution of blisters, environmental factors affecting blistering, extent to which the disease affects normal daily activities and other such details were recorded. In family A the age of onset of blistering was noted to be much later, 6-10 years of age, than that of many Weber-Cockayne families (birth to 18 months of age). The one exception was IV-4 who developed blisters earlier, at 18 months of age, although not when he was learning to crawl. He develops blisters on the abdomen, thighs and feet although they are not much of a problem at present. The earlier onset of blistering in IV-4 may be partly because the family is now more aware of the disease although his mother, III-3, also had blisters as a baby. In general EBS-WC in this family does not present a major problem regarding attendance at school or employment, but the development of blisters does cause pain, distress and prevent them carrying out some normal activities. All members reported blistering on their feet and III-4, II-3 and II-5 blistering on their hands; III-4 and II-3 also develop blisters elsewhere at friction points and when

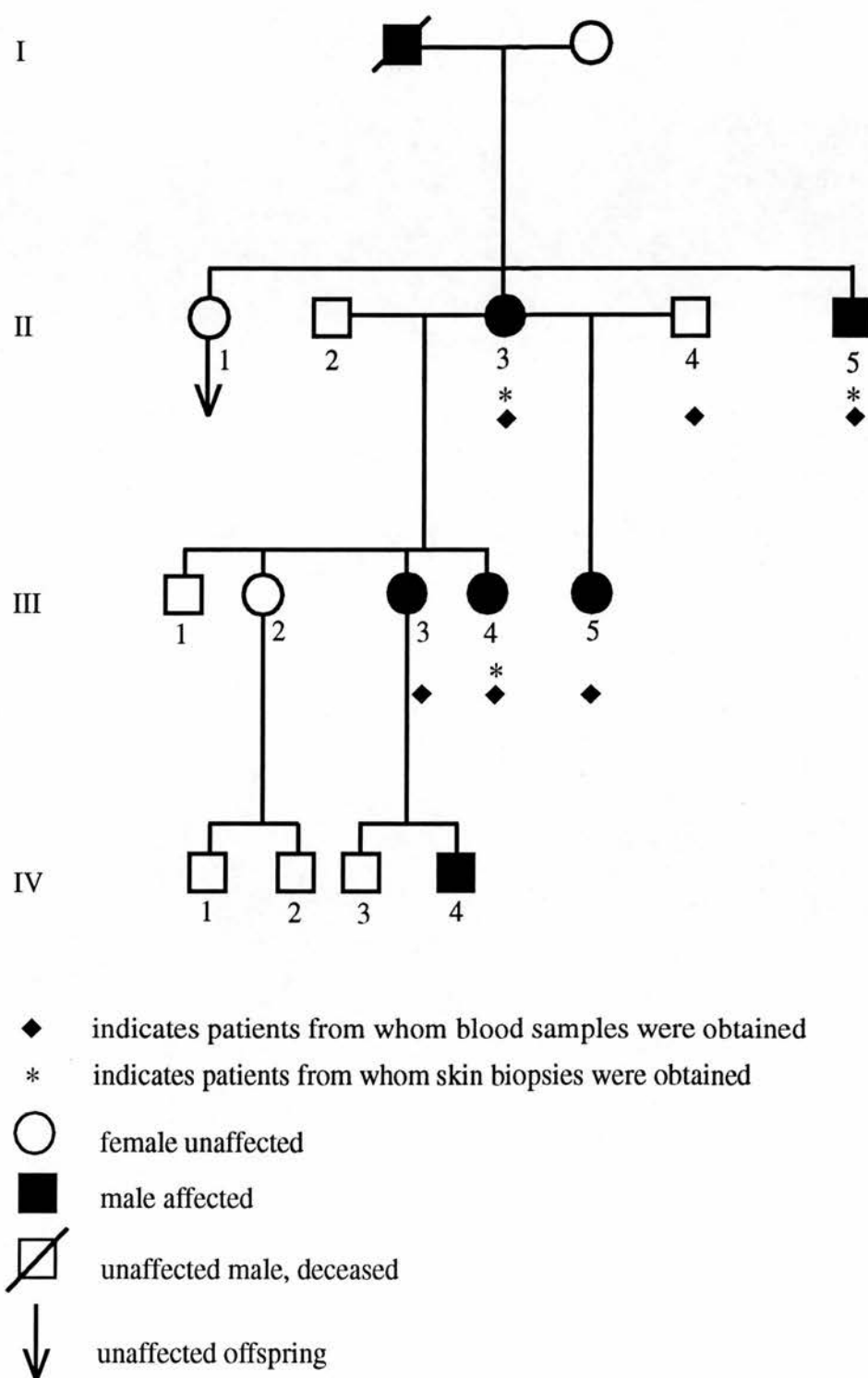


Figure 11. Pedigree of family A

wearing tight clothing. Nail dystrophy was reported in one member. All have the typical EBS-WC seasonal variation, with more severe blistering occurring in the warmer summer months, although they can develop some blisters in the winter. Variation in severity, and in areas of the body affected by the disease within the same family, is a relatively common but unexplained occurrence. Some members may be more likely to avoid activities known to aggravate blister formation.

Keratinocyte Cell Culture

Keratinocytes were cultured from skin biopsies obtained from 3 affected members of the family (II-3, II-5 and III-4) and did not show any abnormal growth characteristics. mRNA was extracted from the cells and reverse transcribed to produce keratin 5 and keratin 14 cDNAs for DNA sequencing. Cytokeratins were also extracted from the cultured keratinocytes for analysis of keratin expression.

Genetic Linkage Analysis

Genomic DNA was extracted from blood samples obtained from five affected members; II-3, II-5, III-3, III-4, III-5 and from one unaffected, related member II-4 for genetic linkage analysis. Analysis using the keratin 8 *Bsa*II and *Mae*II polymorphic markers were not informative in either linking or excluding the disease from the type II keratins (figure 12). However, the disease did not segregate with the keratin 10 *Ava*II polymorphic marker on chromosome 17 (lod score at $\theta = 0$ was -4.54) making it unlikely that any type I keratin carried the defective allele responsible for the disease phenotype (figure 13). Further studies to identify a causative mutation were therefore concentrated on the type II keratin, K5.

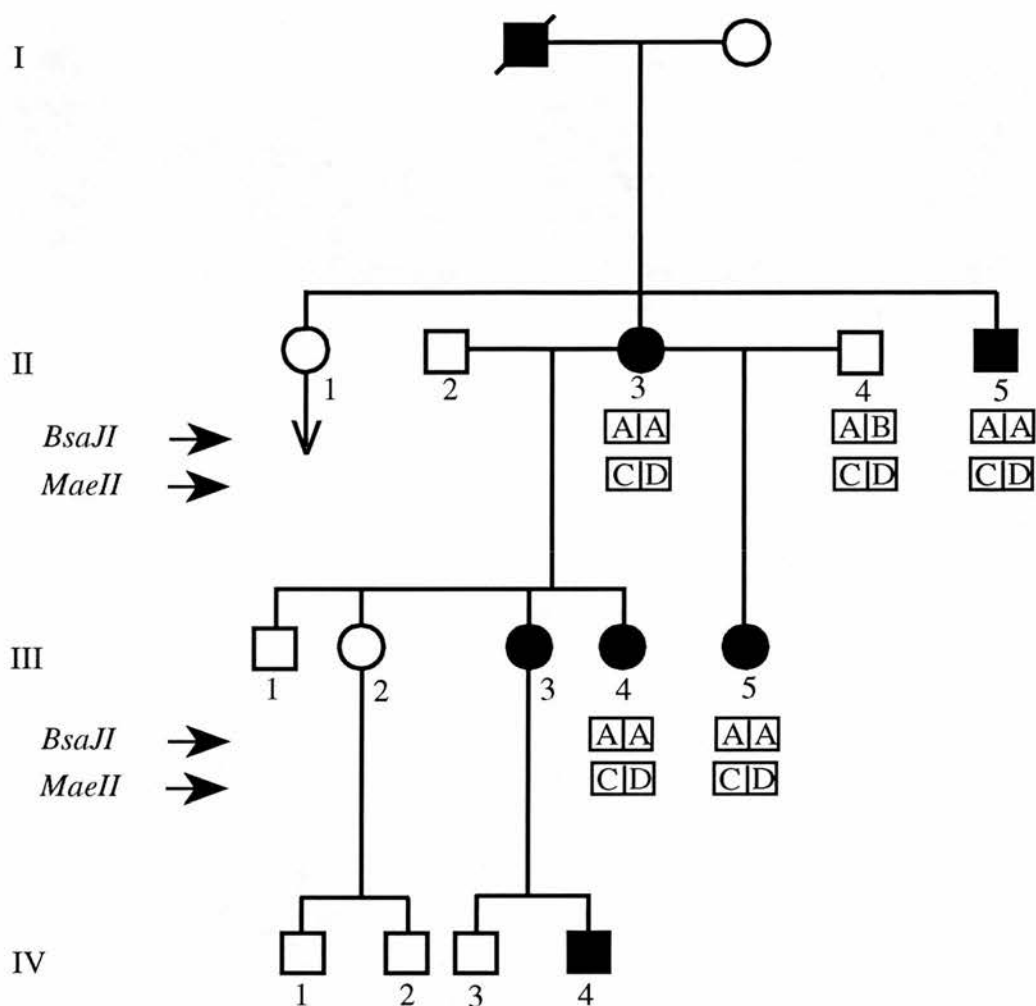


Figure 12. The figure shows analysis of the *Bsa*II (alleles A and B) and *Mae*II (alleles C and D) restriction fragment length polymorphisms in keratin 8. The lod score for *Bsa*II when $\theta=0$ was 0.15 which is not statistically significant.

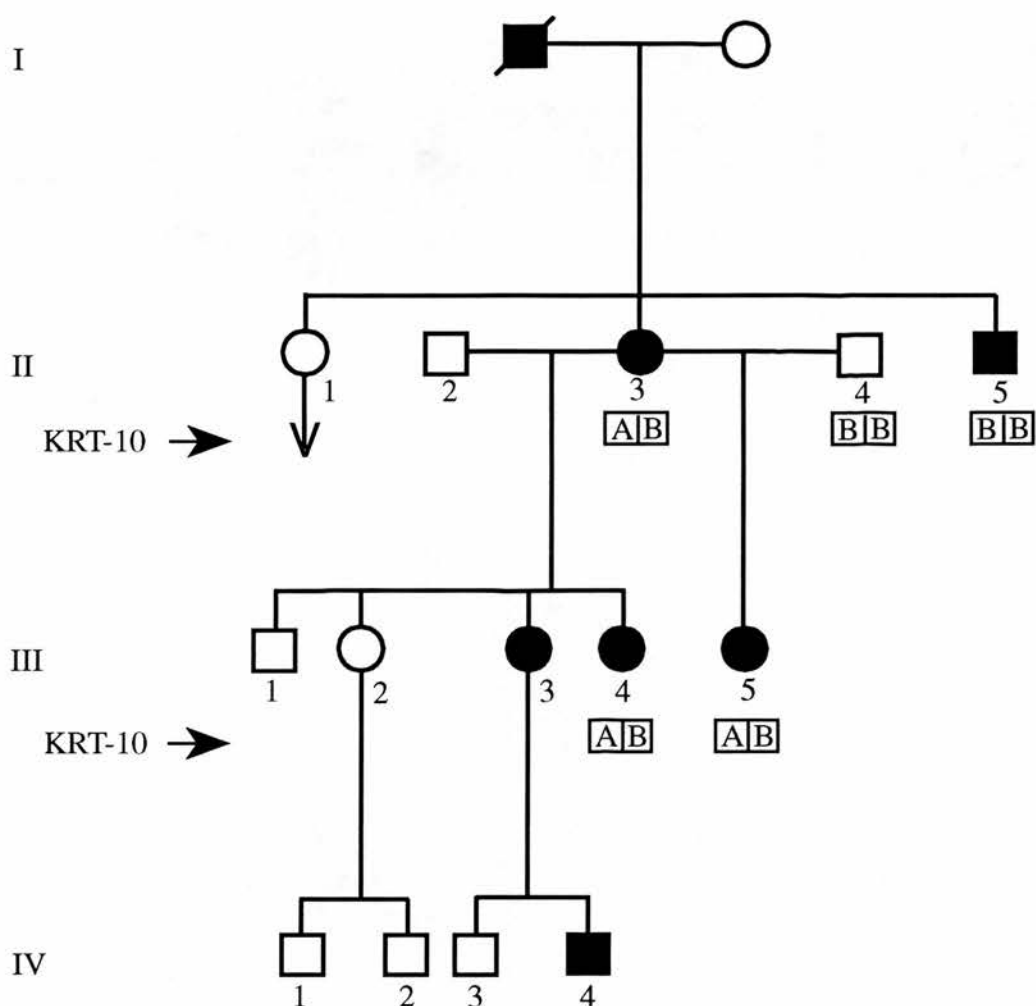


Figure 13. The figure shows exclusion of the disease in family A with chromosome 17 (alleles A and B) using the keratin 10 marker (KRT-10). The lod score at $\theta=0$ was -4.54.

Detection of a Mutation in the L12 Linker of Keratin 5

The highly conserved terminal ends of the rod domain where mutations causing EBS-DM had already been identified (Coulombe *et al.*, 1991a, Lane *et al.*, 1992) were considered to be possible target regions for mutations responsible for the milder phenotypes. However, DNA sequencing of these regions of both keratin K5 and K14, did not reveal any sequence variation from the published sequences. Further analysis, of regions internal to these domains, led to the identification of a point mutation in the L12 linker domain of keratin 5. This was a C-to-T mutation at the first position of codon 331 and resulted in an amino acid change from the predicted arginine to cysteine (figure 14). The L12 domain of several family members was sequenced using both sense and antisense primers and repeated with different PCR products to confirm that this nucleotide substitution was not a PCR or sequencing artifact. The mutation was present in cDNA from II-5 and III-4 (cDNA of II-3 was not sequenced) and in genomic DNA from II-3, II-5, III-3, III-4, and III-5 but was not present in genomic DNA from an unaffected family member (II-4) nor in 4 unrelated genomic DNA samples (3 affected by EBS) or in a further 5 unrelated cDNA samples (all EBS affected) indicating that this mutation was the likely cause for the disease phenotype in this family. All affected members of the family were heterozygous for the mutation, ie. a normal copy of the allele was present as well as the mutant one, but this is not sufficient to prevent keratin disruption as the mutant allele exerts a dominant effect.

No additional amino acid changes were found in the other regions of keratin 5 or keratin 14 that were sequenced (figure 15). However, in keratin 14 a base change of T-to-C at the third position of codon 123 was identified in II-5 (this region was not sequenced from any other members). It does not result in an amino acid change and probably represents a silent polymorphism (Coulombe *et al.*, 1991a). A polymorphism in keratin 5 was also detected in some members of this family (see below, expression of keratins by immunoblotting).

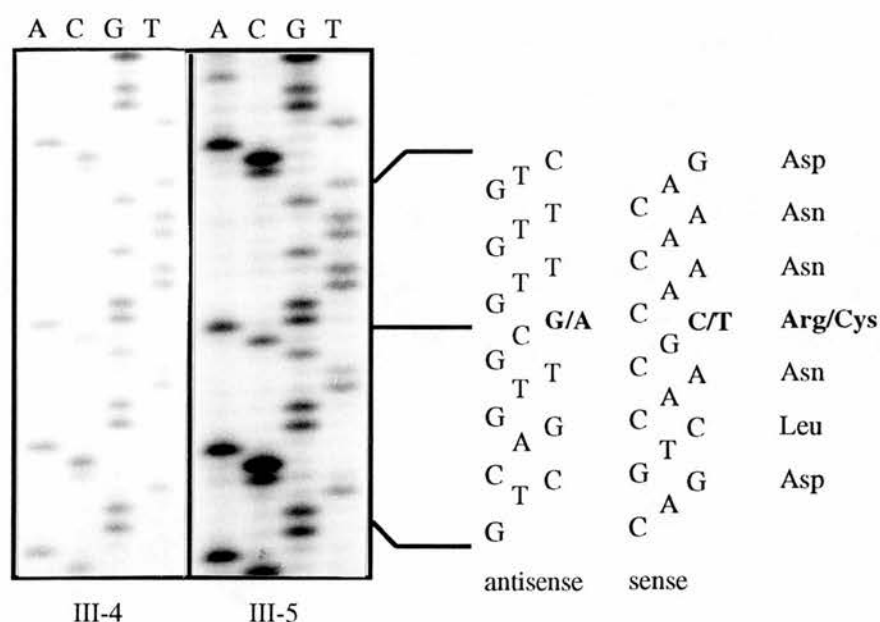
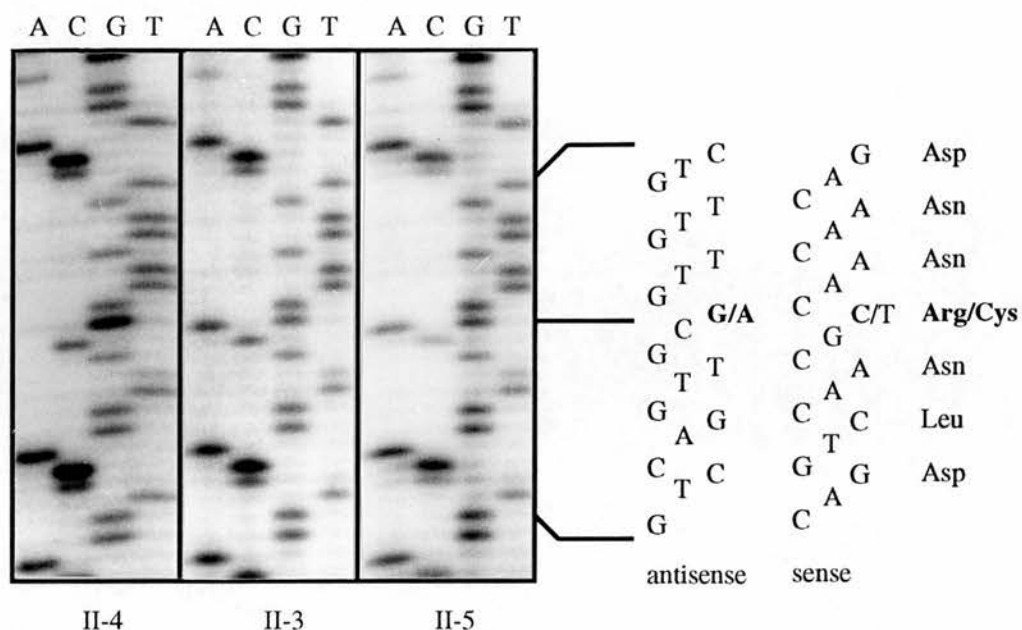


Figure 14. The DNA sequence and predicted amino acid sequence for part of the region encoding the L12 linker domain of keratin 5. The sequence of the PCR-amplified genomic DNA from II-3, II-5, III-5 and cDNA from III-4 of family A show the C-to-T transition resulting in an amino acid change from arginine (Arg) to cysteine (Cys) at codon 331. II-4 is a related but unaffected family member. The termination bases for each lane are indicated by A, C, G and T.

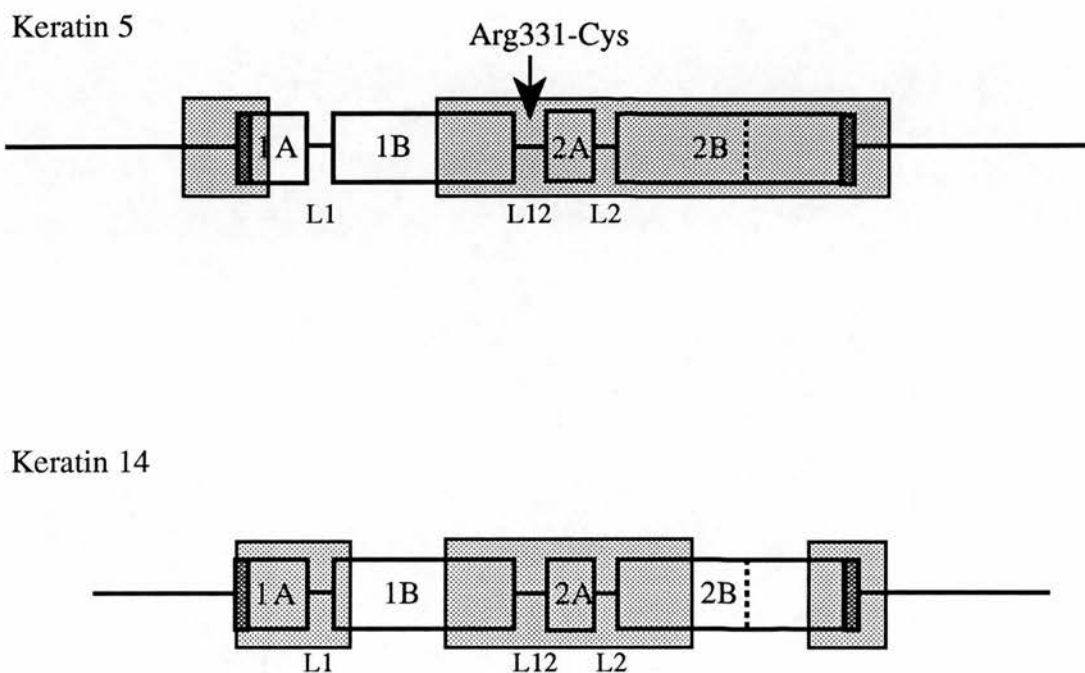


Figure 15. Diagram to show the position of the mutation identified in keratin 5 of family A. The regions of keratin 5 and 14 sequenced from this family are represented by the shaded areas.

In the keratin 5 sequence of II-5 and III-4, codon 387 was represented by serine (TCT) as reported by Lersch *et al.* (1989), and not by threonine (ACA) as reported by Eckert and Rorke (1988). This amino acid variation may represent an error since in all samples sequenced in this study (both affected and unaffected) the amino acid in this position was serine.

Exclusion of Polymorphism

To confirm that this mutation was disruptive to the keratin filament network, resulting in blistering, and was not an innocuous polymorphism occurring within the general population, genomic DNA from 53 unrelated individuals (5 unrelated but EBS affected) and from the 5 affected and 1 unaffected family members was screened. As the arginine to cysteine mutation did not create or destroy a restriction enzyme site, a primer was designed which, in conjunction with the mutation, created a new *AluI* site. This primer was used to amplify a fragment of genomic DNA. The PCR reactions were labelled with $\alpha^{32}\text{P}$ ATP and after digestion with *AluI* were resolved on a 4% sequencing gel and the fragments visualised by autoradiography (figure 16). Genomic DNA from all affected members was cut at this new site, by *AluI*, resulting in an additional fragment in these samples. Genomic DNA from the unaffected relative (II-4) and 53 controls were not cut at this site. It is highly probable that this mutation of arginine to cysteine is responsible for the disease phenotype of this family (χ^2 (1d.f.)=46.02; $P<0.001$).

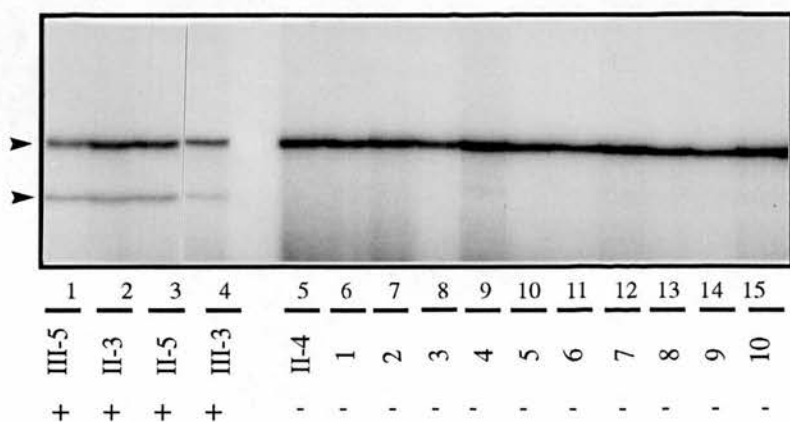


Figure 16. Autoradiograph showing a PCR-amplified fragment of genomic DNA of keratin 5 from four affected members of family A, III-5, II-3, II-5, III-3 (lanes 1-4); an unaffected related member, II-4 (lane 5) and 10 unrelated, unaffected controls (lanes 6-15) for the presence of a new *AluI* site. The arrows indicate the normal fragment (top) and the fragment produced after digestion with *AluI* (bottom). EBS affected patients are indicated by + and unaffected family members or controls by -.

Expression of Keratins by Immunoblotting

Cytoskeletal extracts from the cultured keratinocytes were examined for keratin expression by SDS polyacrylamide gel electrophoresis (10% acrylamide gels) and immunoblotting with anti-keratin antibodies. No obvious abnormality or deficiency was detected in the pattern of keratins expressed compared to those of normal keratinocytes. There were no additional bands present and the keratins migrated at the same rate as those extracted from unaffected control cells. As expected, the major keratin pairs expressed were the basal cell keratins, K5 and K14, together with K6 and K16 that are expressed by cultured cells. Some members of the family were found to be polymorphic for the K5a/K5b allele, detected by staining of a doublet band with the monoclonal antibody AE14 to keratin 5 (see Keratin 5a/5b Polymorphism). The keratins were detected by staining the immunoblots with the following antibodies: AE14 to K5, RCK102 to K5 and K8, PCK26 to K1, K5, K6 and K8, α IFA to most intermediate filaments, AE3 to type II keratins, AE1 to type I keratins, LLOO1 to K14 (figure 17a-f). Small amounts of K1, K10 and K17 were detectable but there was no reactivity with antibodies to K8 (LE41), K18 (LE61) or K19 (LP2K). TR146 cells differ from the control and EBS keratinocytes as they express K17 instead of K16 and also small amounts of K8, K18 and K19 (Rupniak *et al.*, 1985).

Keratin 5a/5b Polymorphism

When immunoblots were stained for keratin 5 with the AE14 antibody a doublet band was visible in two of the three affected individuals (II-3 and II-5) indicating that the family was polymorphic for keratin 5. The normal human keratin 5 gene is polymorphic with two co-dominant alleles, K5a and K5b, detectable by their different electrophoretic migration rates. K5a is represented by the higher slower migrating band and K5b by the lower faster band. These alleles have been shown by Mischke *et al.* (1990) to be inherited in Hardy-Weinberg equilibrium and are transmitted as Mendelian traits.

Figure 17. Immunoblots of cytoskeletal extracts from primary keratinocyte cultures from EBS affected members of family A, an unaffected unrelated control and TR146 cells, stained with anti-keratin antibodies. Samples from EBS affected individuals are indicated by + and unaffected controls by -. Prestained molecular weight markers (std) were used to estimate the molecular weight: phosphorylase B = 106 kD; bovine serum albumin = 80 kD; ovalbumin = 49.5 kD; carbonic anhydrase = 32.5 kD. The prestained markers run at different molecular weights than the original proteins due to the covalently attached dye.

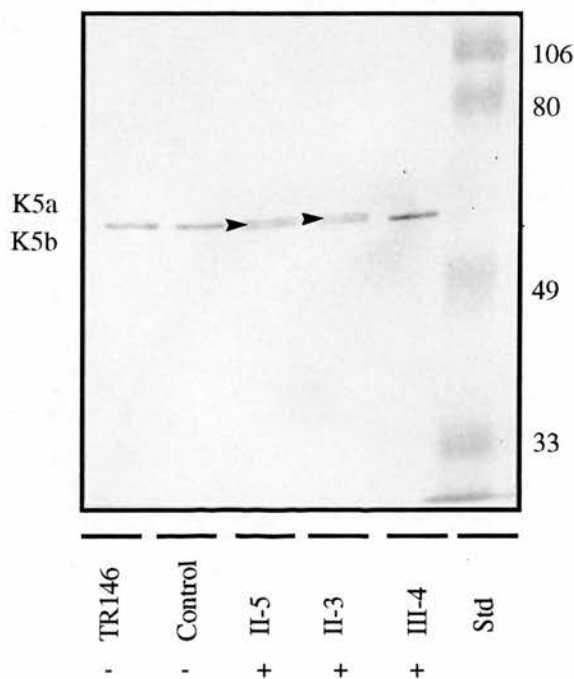


Figure 17a. Stained with AE14, a monoclonal antibody specific to keratin 5. The arrows indicate the K5a/K5b polymorphism present in II-3 and II-5. The K5a allele is represented by the higher, slower migrating band and the K5b allele by the lower, faster migrating band. III-4, control keratinocytes and TR146 cells were homozygous for the more common K5b allele.

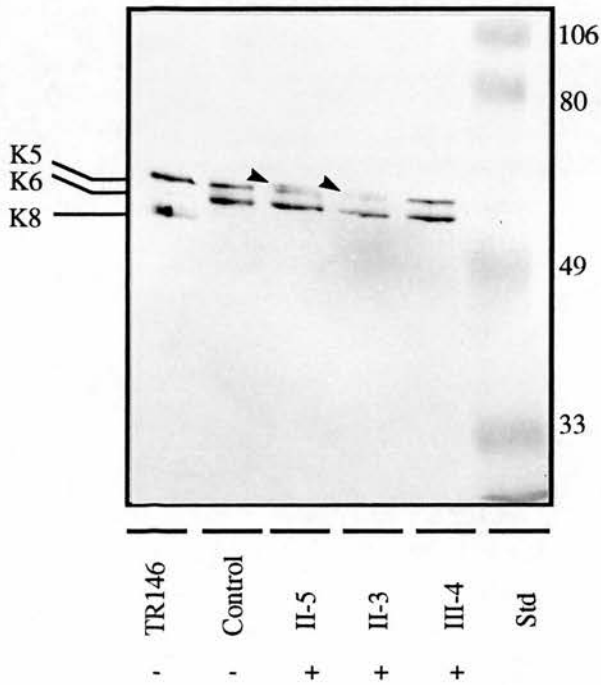


Figure 17b. Stained with RCK102 to the type II keratins K5 and K8. The K5a/K5b polymorphism was detected in II-3 and II-5 as indicated by the arrows. K8 was only expressed by the TR146 cells. There was some cross reactivity, with staining of K6 in II-5, II-3, III-4 and the control keratinocytes.

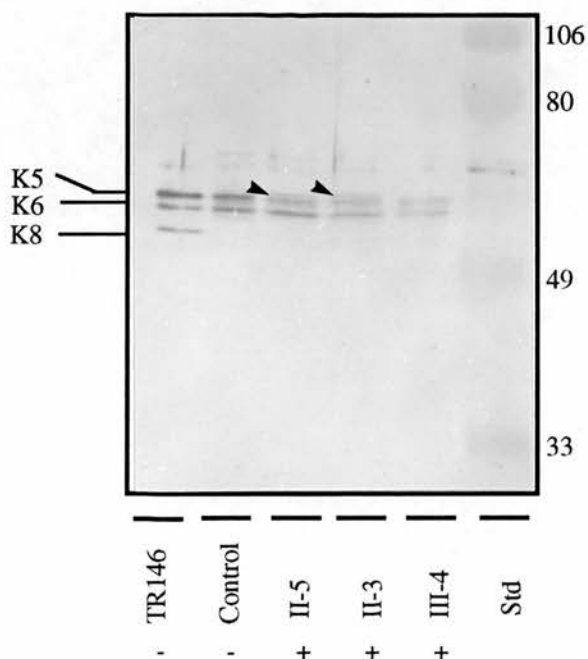


Figure 17c. Stained with PCK26, an antibody that reacts with the type II keratins, K1, K5, K6 and K8. K5 (with the K5a/K5b polymorphism detectable in II-3 and II-5, indicated by the arrows) and K6 were expressed by the cells in approximately equal amounts with, as expected, only small amounts of K1. The TR146 cells also stained for K8.

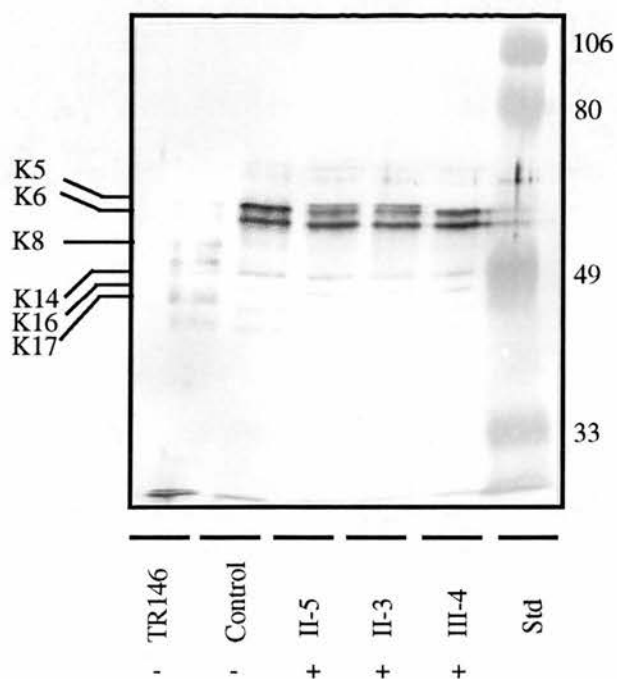


Figure 17d. Stained with α IFA, an antibody to all intermediate filaments. α IFA reacts more strongly to the type II keratins by immunoblotting than to the type I keratins, as shown by the stronger staining of K5 and K6 compared to that of K14 and K16. In addition the TR146 cells expressed K8 and, K17 instead of K16. The K5/K5b polymorphism was again detected in II-3 and II-5.

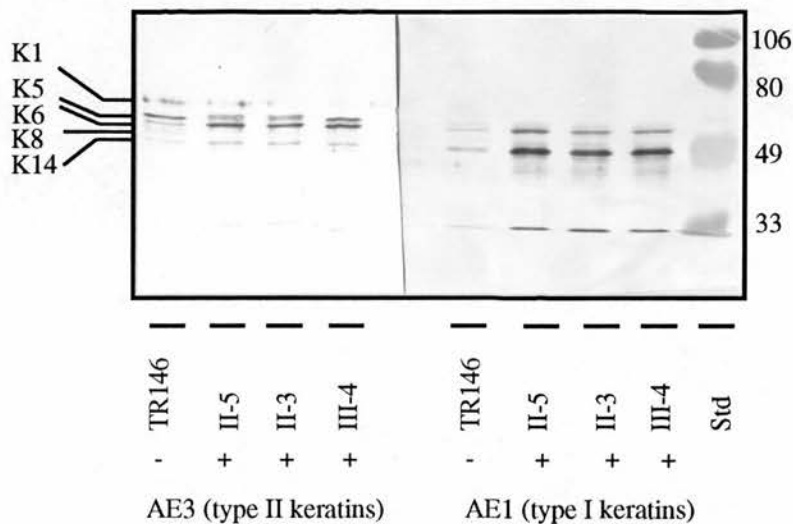


Figure 17e. Stained with AE3 to all type II keratins. Keratins K5 and K6 were detectable in all the samples with trace amounts of K1, and additionally K8 in the TR146 cells. The K5a/Kb polymorphism was also detected in II-3 and II-5. There was slight cross reactivity with some staining of K14.

With AE1, which reacts to all type I keratins, K14 was found, as expected, to be the major type I keratin expressed by the cells. There was also cross reactivity with some staining of K6.

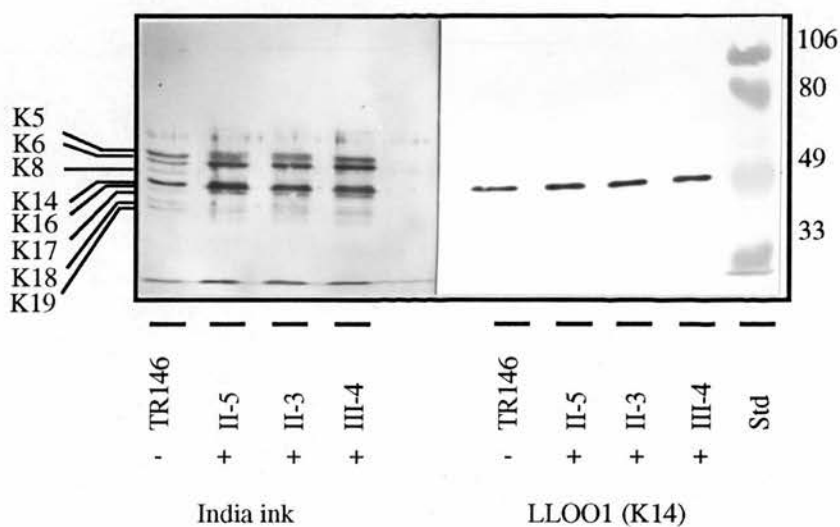


Figure 17f. Stained with India ink which stains all proteins; the predominant keratins expressed by the EBS keratinocytes were K5, K6, K14 and K16 with trace amounts of K1 and K10. The TR146 cells expressed K5, K6, K14, K17 rather than K16 and small amounts of K8, K18 and K19.

All the extracts showed a normal staining pattern with LLOO1, a monoclonal antibody specific to the type I keratin, K14.

The lower K5b allele is reported to occur in approximately 84% of the general population, the K5a allele in 1% and the heterozygous K5a/5b allele in about 15% of the population (Mischke *et al.*, 1990). The Hardy-Weinberg law can be used to calculate the frequency of alleles within a population. If p represents the frequency of the dominant allele and q the frequency of the recessive allele then $p + q = 1$. A population adhering to this law will transmit the same frequencies of alleles to the next generation; these ratios can be calculated using a "Punnett square" where $p^2 + 2pq + q^2 = 1$.

DNA sequence analysis of the keratin 5 by Wanner *et al.* (1993) identified 3 alleles for the K5 locus, in the H1 subdomain. All are single nucleotide substitutions and one of them, G-to-A, changes the amino acid sequence from glycine (uncharged) to glutamic acid (charged) which alters the charge of the amino acid thereby explaining the different migration rates on SDS polyacrylamide gels. Wanner *et al.* (1993) also noted that there were no sequence variations in the rod domain or in the carboxyl terminal domain of keratin 5. The latter is a region where differences in the V2 subdomain of keratin 1 and 10 have been found (Korge *et al.*, 1992a, b). DNA sequencing of the H1 subdomain of keratin 5 (genomic DNA) confirmed that those showing the protein doublet band (II-3 and II-5), were heterozygous for the K5a/K5b allele, while a further three affected members (III-3 (not shown), III-4 and III-5) and one unaffected (II-4) member of the family were all homozygous for the more common K5b allele (figure 18). This polymorphism did not therefore segregate with the EBS phenotype in this family.

This data was used to apply the Hardy Weinberg Law; in the first generation 33% of the alleles were the K5b allele ($p = 0.33$) and 66% were the heterozygote K5a/K5b ($pq = 0.66$) and in the second generation 100% of the alleles were K5b ($p = 1$). Although the results suggested that the alleles were not inherited in the Hardy-Weinberg ratios much larger sampling numbers would be required to obtain significant values.

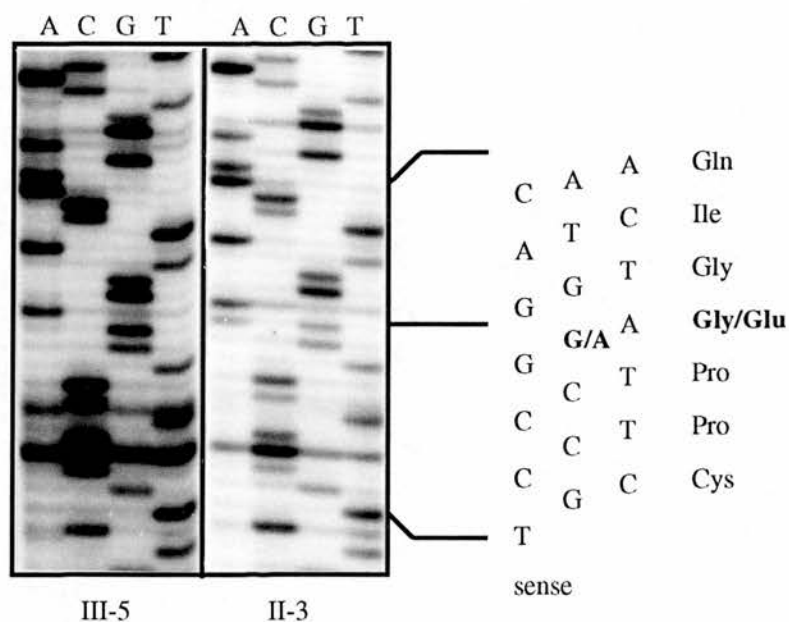
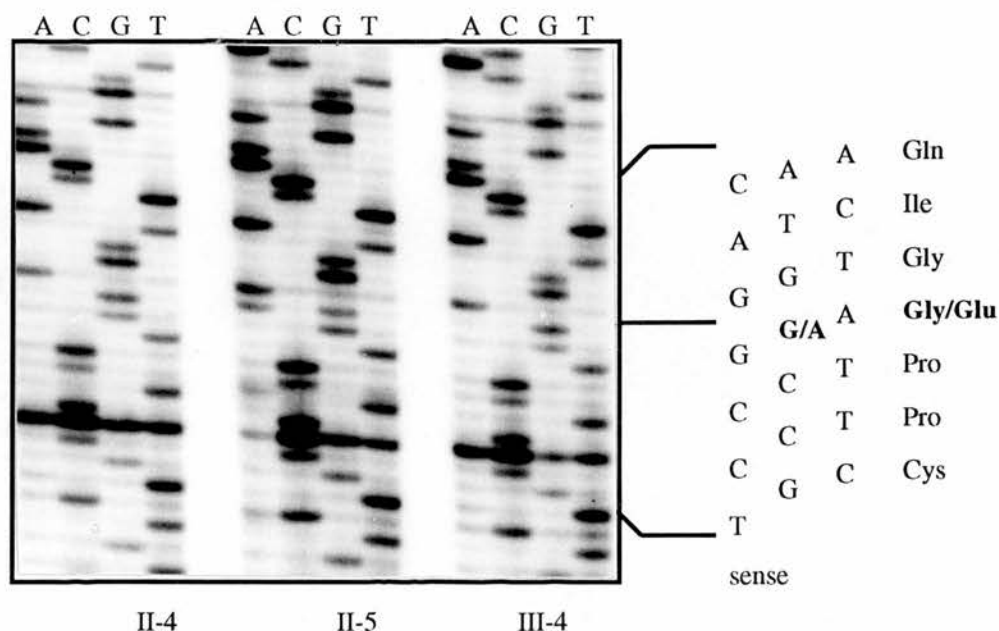
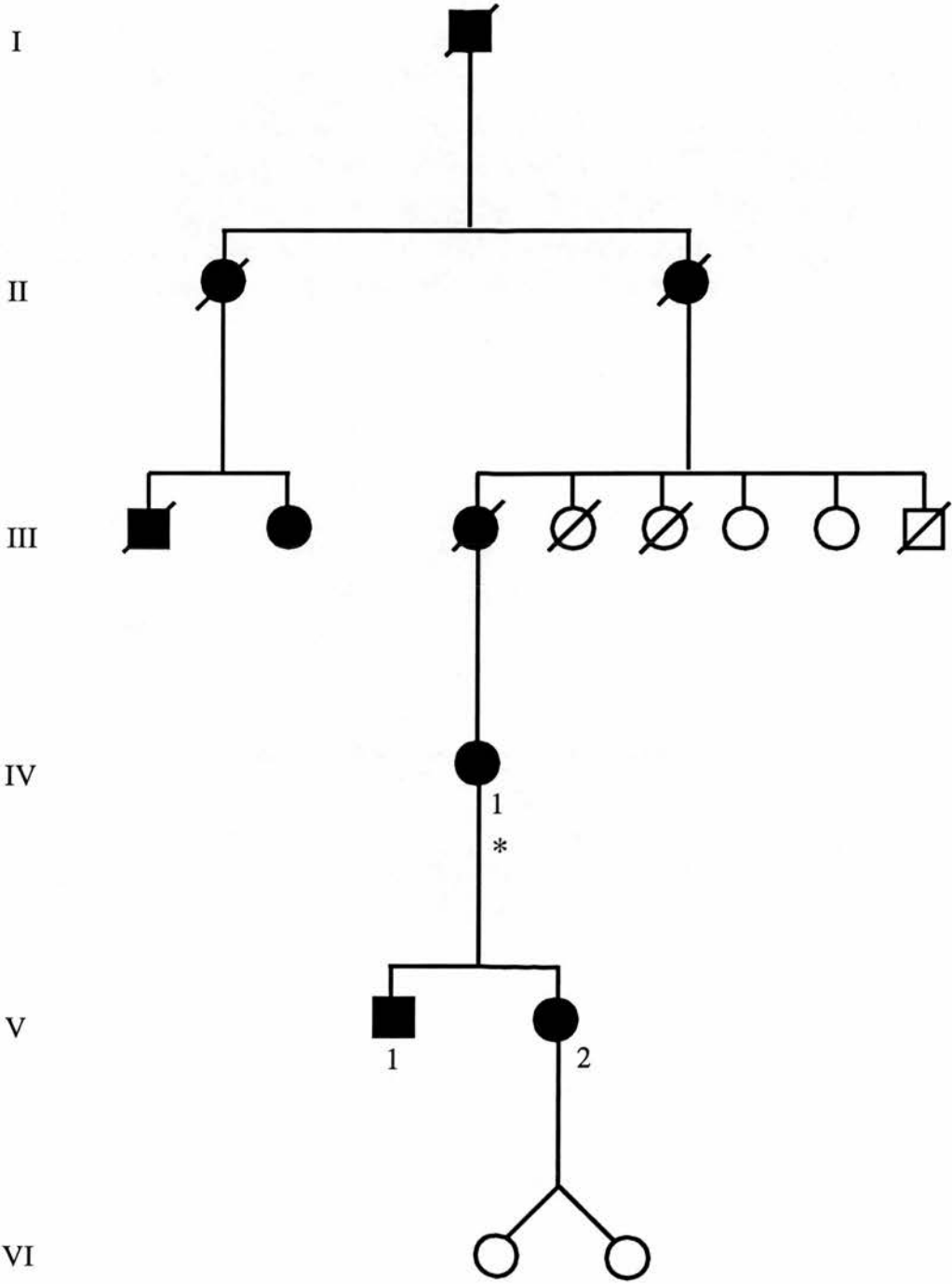


Figure 18. The DNA sequence and predicted amino acid sequence for part of the region encoding the head domain of keratin 5. The sequence of PCR-amplified genomic DNA from II-4 (unaffected), II-3, II-5, III-4 and III-5 (all affected) of family A. The K5a/b polymorphism at codon 138 was present in II-3 and II-5, shown by a glycine (Gly) to glutamic acid (Glu) amino acid change. II-4, III-4 and III-5 were all homozygous for the K5b allele, represented by glycine (Gly). The termination bases in each lane are indicated by A, C, G and T.

4.2.2 Family B



* indicates patient from whom a skin biopsy was obtained

Figure 19. Pedigree of family B

Clinical Description of Family B

Two affected members (IV-I and V-2) of this small kindred (figure 19) were interviewed for the UK National EB Register. Both developed blisters between the age of 7 months to 2 years of age. As well as on the hands and feet, blisters develop at other sites including the ankles, thighs, and waist. Blisters are induced by tight clothing, plasters, the heat from ironing, dancing and walking. In both, blistering is much more severe in the summer and patient V-2 avoids shopping in the city centre at this time of year because of blisters developing on the feet due to the heat and friction while walking. This member of the family also developed blisters on her back due to prolonged bed rest in hospital. The youngest generation (VI) although shown on the pedigree as being unaffected may be affected as some blistering around the nappy area has now been observed.

Keratinocyte Cell Culture

Keratinocytes were cultured from a skin biopsy obtained from IV-I. Staining of the cells for keratin 5 and keratin 14 by immunofluorescence, although very weak, indicated that the keratin filament network was apparently normal. Cytokeratin extracts were examined for keratin expression and, mRNA was extracted and reverse transcribed to produce keratin 5 and keratin 14 cDNAs for DNA sequence analysis.

Identification of a Mutation in the L12 Linker of Keratin 14

No sequence variations were found in the highly conserved terminal ends of keratin 14 or in the carboxy terminal end of keratin 5. Subsequent sequence analysis of internal regions of the keratin rod domain revealed a point mutation in keratin 14 at codon 270 in the L12 linker domain. This G-to-A nucleotide substitution at the first position of the codon resulted in an amino acid change from the predicted valine to a methionine (figure 20). This mutation was present in cDNA, from IV-I, sequenced with both sense and antisense primers. There were no other sequence variations in the regions of

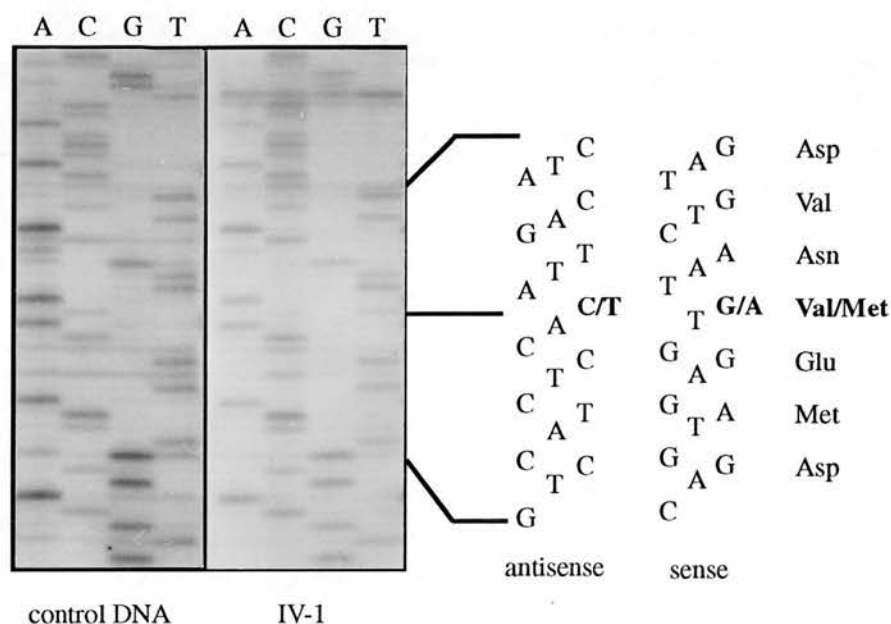
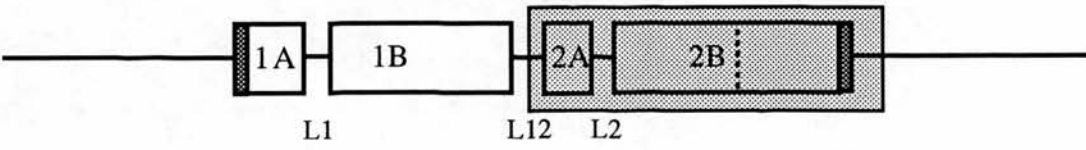


Figure 20. The DNA sequence and predicted amino acid sequence for part of the region encoding the L12 linker domain of keratin 14. The sequence of PCR-amplified keratin 14 cDNA from IV-1 (EBS affected) of family B and from an affected but unrelated individual. The G-to-A transition present in IV-1 results in an amino acid change of valine (Val) to methionine (Met) at codon 270. The termination bases for each lane are indicated by A, C, G and T.

Keratin 5



Keratin 14

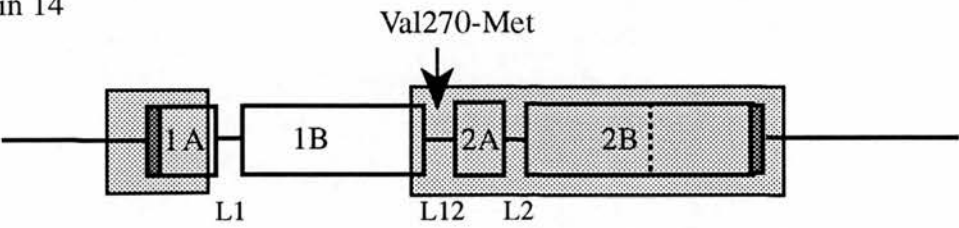


Figure 21. Diagram to show the position of the mutation identified in keratin 14 of family B. The shaded areas represent the regions of keratin 5 and keratin 14 sequenced from this family.

keratin 5 or keratin 14 that were sequenced (figure 21).

This same nucleotide substitution, at codon 270, has been identified in another EBS-WC family (Rugg *et al.*, 1993a). This very large Scottish family has been affected by blistering for at least five generations, inherited in an autosomal dominant manner.

Three members of this family were originally presented as separate families, but after identical mutations were found and further interviews with the families it was deduced that the patients were distantly related. However, from the data available it appears that this smaller family is not related. This L12 region of keratin 14 was also sequenced from a further 8 unrelated cDNA samples (5 affected by EBS) and 4 unrelated genomic DNA samples (3 affected by EBS), none of which had this mutation.

Screening for the Mutation

Control DNA was screened using PASA analysis (PCR amplification of specific alleles) to exclude the possibility that this mutation was an innocuous polymorphism in the general population. This method required an exact match of the 3' base of the primer for amplification of the DNA by PCR to occur. The mutant base was set at the 3' position of one primer so that only samples with the mutant allele were amplified and, the normal wild type base was at the 3' position of a second primer so all the samples were amplified. Two PCR reactions were set up for each of the 55 unaffected, unrelated control genomic DNAs and the cDNA from the affected patient (IV-I), each with one of these primers and plus another primer. After PCR amplification the samples were resolved on a 1.5% agarose gel. The cDNA from the affected patient (IV-1) produced a fragment of 130 base pairs with both the mutant and wild type primers, whereas the control genomic DNAs were only amplified with the wild type primer to produce a fragment of 130 base pairs, indicating that none of these samples

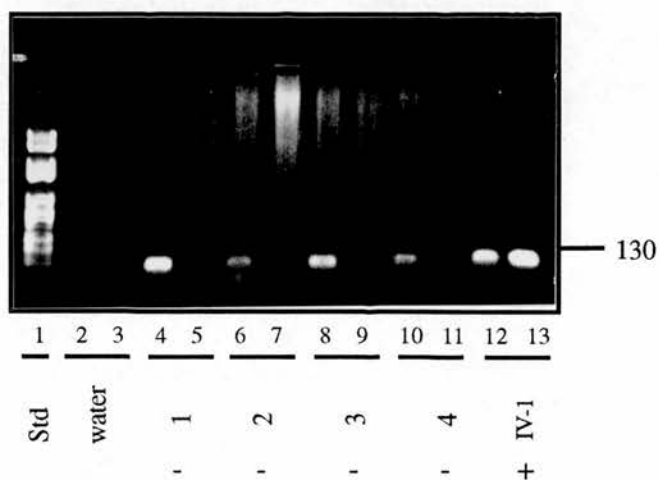


Figure 22. Ethidium bromide staining of a 1.5% agarose gel showing PASA analysis of genomic DNA for the G-to-A mutation in the L12 linker domain of K14. Amplification of a 130 base pair fragment of DNA from four unrelated unaffected individuals is shown in lanes 4-11, and IV-1 (EBS affected, family B) in lanes 12-13 using the wild type primer (even numbers) and the mutant primer (odd numbers). DNA molecular weight markers (std) were used to estimate the molecular weight of the fragments.

contained the mutant base (figure 22). It is therefore highly unlikely that this valine to methionine amino acid change is a common polymorphism (χ^2 (1d.f.)=55; $P<0.001$).

Keratin Expression Detected by Immunoblotting

The cytokeratin extracts were analysed by SDS polyacrylamide gel electrophoresis (12% acrylamide gels) and immunoblotting with anti-keratin antibodies. There was no detectable abnormality in the pattern of keratin expression or difference in the migration rate of the proteins extracted from the keratinocytes of IV-I compared to those from control cells. The antibodies used to detect the keratins were LLOO1 to K14, RCK102 to K5 and K8, α IFA to all intermediate filaments, AE3 to type II keratins, AE1 to type I keratins, and PCK26 to K1, K5, K6 and K8 (figure 23a, b). Staining with α IFA, RCK102 and AE3 showed a doublet band for K5 indicating that IV-I could be polymorphic for the K5a/K5b allele. This region of K5 has not been sequenced to confirm this.

Figure 23. Immunoblots of cytoskeletal extracts stained with anti-keratin antibodies to show the keratins expressed by a primary keratinocyte culture from IV-1 (affected by EBS) of family B and from TR146 cells (control). Prestained molecular weight markers (std) were used to estimate the molecular weight: phosphorylase B = 106 kD; bovine serum albumin = 80 kD; ovalbumin = 49.5 kD; carbonic anhydrase = 32.5 kD.

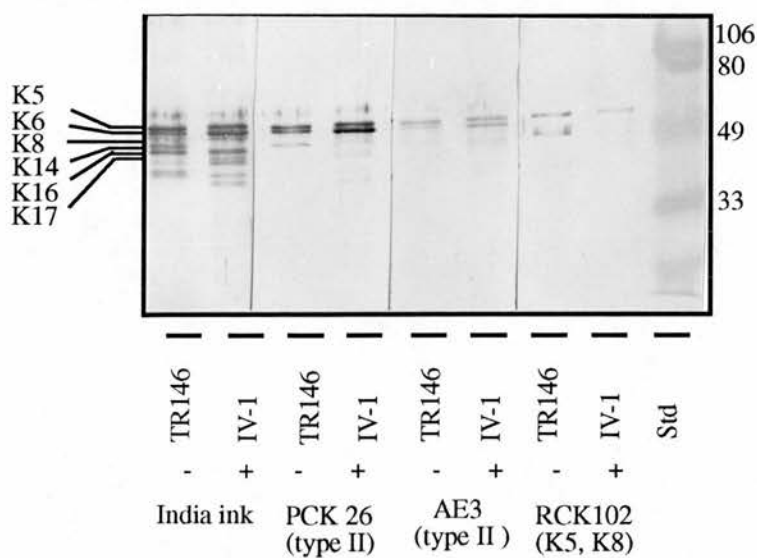


Figure 23a. India ink staining to show all the proteins expressed by the cultured cells. Staining with PCK26 (to type II keratins), AE3 (to type II keratins) and RCK102 (to keratins K5 and K8) showed that K5 and K6 were the predominant type II keratins expressed by both cell cultures, with some K8 expressed by the TR146 cells.

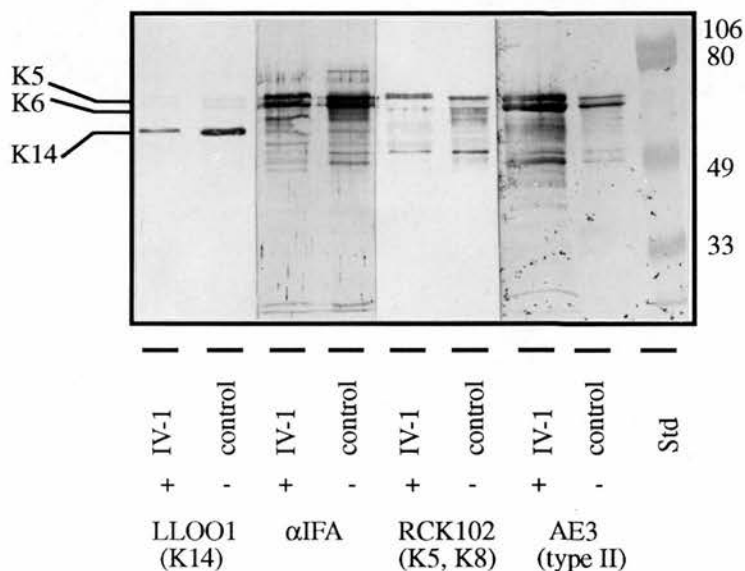


Figure 23b. Stained with LL001, specific to keratin 14, αIFA to all intermediate filaments, RCK102 to keratins K5 and K8 and AE3 to type II keratins.

IV-1 may be polymorphic for the K5a/K5b polymorphism as there appears to be a doublet band stained for K5. However, this is not obvious on the previous blot (figure 23a), and this region of K5 has not been sequenced to confirm whether this individual is heterozygous for the K5a and K5b alleles.

Discussion

The identification of these two mutations in the central L12 linker region was surprising. Relatively little is known about this domain of intermediate filaments and its role in filament assembly and network formation and, until now, it has never been considered particularly important in the proper functioning of the cell cytoskeleton. Analysis, using algorithms which predict the secondary structure of proteins, suggest that the L12 linker domain is highly flexible and non-helical. This is due to the lack of heptad repeats and the presence of proline and glycine residues which are predicted to destabilize α -helices and to disrupt potential coiled coil interactions. Due to the sequence of (polar-apolar)₄ residues, the L12 linker domain has the potential to form a β sheet structure. It is known, that throughout most intermediate filaments, this linker domain is well conserved both in number and position of amino acid residues (Steinert & Parry, 1985). This would imply that it is an important structural feature and that sequence variation, as identified in these EBS patients, is detrimental to the integrity of the cell cytoskeleton. Figure 24, shows the sequence alignment of the L12 linker domain for type I and II keratins and the position of these EBS-WC mutations. The position of a mutation that has since been reported in this region of keratin 14 in a family with EBS-K is also shown (Humphries *et al.*, 1993).

The majority of deletion and point mutagenesis studies have concentrated on the role of the α -helical regions of the rod domain, particularly the terminal ends and the few experimental studies analysing the L12 linker domain of intermediate filaments have so far failed to determine its role in filament assembly or network formation. Gill *et al* (1990), experimentally increased the length of the L12 domain in a neurofilament but there was no major disruption to the filaments. McCormick *et al.* (1991) constructed hybrids of keratin 14-vimentin rod domains using the linker regions as the splice sites and expressed them in BHK cells, containing vimentin, in MCF-7 cells containing keratin, and in PtK2 cells that express both vimentin and keratin networks. Hybrids with a L12 region that was half keratin linker and half vimentin linker did, when expressed in cells, show aberrant intermediate filament formation but it is not clear whether this was due to the altered L12 or to the switched rod domains or a combination of both. Although the keratin-vimentin hybrids did disrupt either the vimentin, the keratin or both the filament networks, this swapping of the α -helical domains was not as deleterious as actually deleting portions of the rod domain. More specifically, the effects of individual amino acids in this region were demonstrated by Letai *et al.* (1992). They substituted the proline and glycine residues in the L12 linker of keratin 14 to increase the α -helicity. Expression of these mutants in epithelial cells (SCC-13 or PtK2) did not result in any observable effect on keratin filament formation, at least not under tissue culture conditions, although there was a slight tendency for filaments assembled *in vitro* with wild type keratin 5 to aggregate. Assuming that the amino acid substitutions did favour an α -helical conformation, these results indicated that the apparently normal flexible nature of this domain is not a major requirement for 10 nm filament assembly, at least *in vitro*. Collectively the results from all these studies predict that mutations leading to disruption of the keratin filament network would be more likely to occur in the α -helical domains of the rod than in the non-helical linker domains.

However, the mutations identified in these EBS-WC families provide evidence that the L12 linker domain does play an important role in maintaining the overall structural integrity of the cell cytoskeleton. Due to the subtleties of these mutations, it is very difficult to carry out functional assays *in vitro* to demonstrate the effects of the mutations on filament assembly and network formation. In the more severe form of EBS, Dowling-Meara, the characteristic abnormal tonofilament clumping has been experimentally reproduced in epithelial cells transfected with constructs containing identified mutations. Since basal keratinocytes of EBS-WC patients show few, if any, ultrastructural abnormalities (Haneke & Anton-Lamprecht, 1982), similar functional assays, as applied to EBS-DM, would be unlikely to show any effect of an EBS-WC causing mutation. Therefore, although it may not be possible to demonstrate experimentally that these single amino acid substitutions disrupt the keratin network, keratin mutations identified in EBS-WC are concluded to be causative for the disease if they satisfy several criteria. If the mutation is only carried by the affected members of a family and not in 100 unrelated control alleles, if the disease and therefore the mutation is inherited in an autosomal dominant manner and if genetic linkage data, where informative, supports the findings.

Analysis of the normal and mutant amino acid sequences using algorithms can be used to predict the effect of the mutant sequence on the secondary structure of the keratin filament. Using the conformational information from the determination of crystal structures of globular proteins, algorithms can predict the most likely secondary structure for any particular piece of sequence (Parry, 1990). This may show how the mutation would affect the overall integrity of the cytoskeleton. The L12 domain for both the normal and mutant keratin 5 and 14 was analysed using Gene Works version 2.1 (Intelligenetics Inc.). Two algorithms were used (Garnier *et al.*, 1978; Chou & Fasman, 1978) and the results combined to increase the accuracy. The valine270 to methionine mutation in the L12 linker of keratin 14 of family B was predicted to

increase the α -helicity. This could potentially alter the length or the flexibility of the domain. Similar analysis of the arginine331 to cysteine mutation in keratin 5 of family A did not predict any obvious structural change that might alter the strength or flexibility. However, the introduction of a cysteine in place of an arginine in the amino acid sequence is potentially very disruptive due to the difference in size and its capacity for cross-linking.

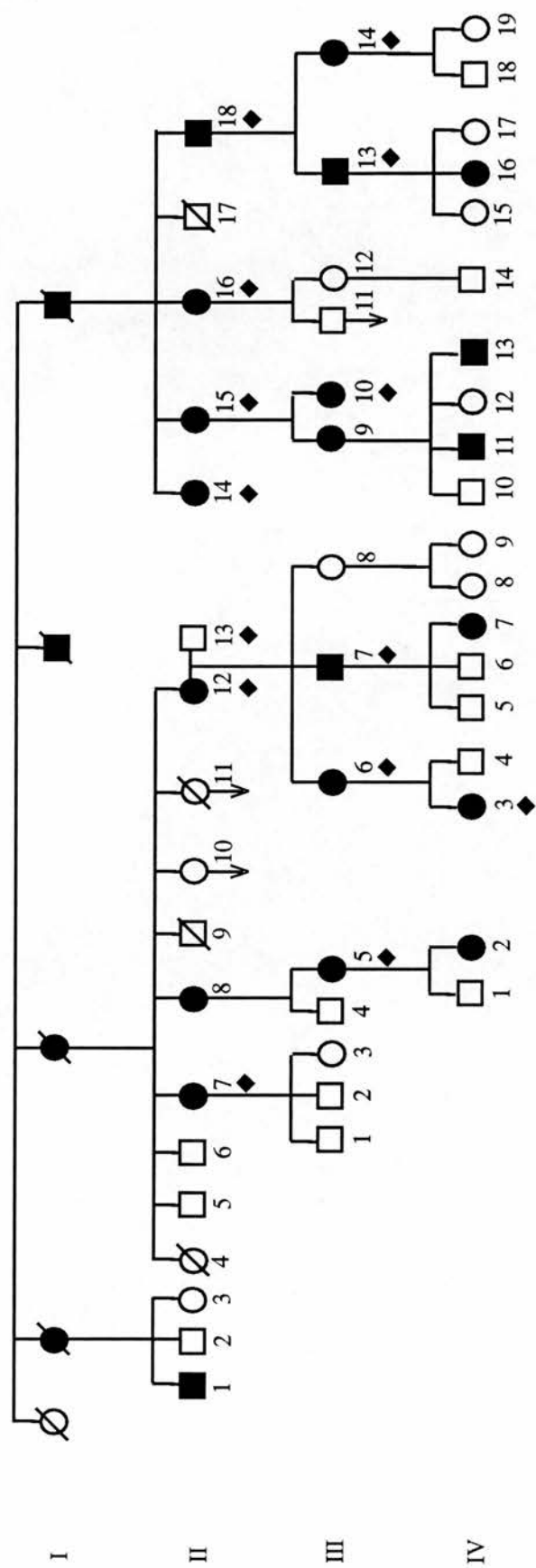
As already mentioned, since the identification of these mutations another point mutation has been reported in the L12 linker region of keratin 14 (Humphries *et al.*, 1993). The methionine to arginine amino acid change at codon 272 in this family is within two residues of the valine to methionine mutation reported here, but results in a more severe disease phenotype with generalised blistering typical of EBS-K. This further demonstrates that the rod domain, and subdomains within it, are not uniform in their role in the keratin network; individual residues vary in their importance to the overall functioning of the cell cytoskeleton.

4.3 Family C: A Point Mutation in the 1A Domain of Keratin 5

A study of this large Scottish family (figure 25) affected by the Weber-Cockayne variant of EBS led to the identification of a point mutation in the 1A domain of keratin 5 which resulted in an amino acid change from asparagine193 to lysine.

Clinical Description

Blistering has been inherited in an autosomal dominant manner for at least four generations in this family. Eighteen affected family members were interviewed for the UK National EB Register (those from whom blood samples were taken and II-8, III-9, IV-2, IV-7 and IV-16). A marked seasonal variation in the severity of the disease was reported by all members, with more severe blistering in warmer weather, although some do develop blisters during the winter. Blisters appeared within the first six months and for some there has been an improvement with age. All patients reported blistering of the feet and all except II-18 and III-14 develop hand blisters (figure 26). Blistering at sites other than the hands and feet is common in all except III-7 and III-10. There is quite a wide variation in severity between members of this family and a point of interest was that no mouth blisters were reported in this large family. The effects of moist sea air in reducing blistering was noted by IV-3 who normally develops blisters of moderate severity during warmer weather on the hands, feet, legs (under ski boots), and buttocks, but while on holiday in Teneriffe she did not develop blisters even though she did quite a lot of walking.



◆ indicates patients from whom blood samples were obtained

Figure 25. Pedigree of family C



Figure 26 Soles of patient IV-3.

Detection of a Mutation by DNA Sequencing

Genomic DNA was extracted from blood samples from 13 affected members (see figure 25) and 1 unaffected member (II-13) for DNA sequencing. A C-to-G base substitution at the third position of codon 193 was identified in the 1A domain of keratin 5 which resulted in an amino acid change from the predicted asparagine to lysine (figure 27). For this region, both strands of DNA were sequenced, and the mutation was identified in genomic DNA sequenced from 5 affected family members II-12, II-14, III-5, III-14, IV-3 but was not present in 4 unrelated genomic DNA samples (2 affected by EBS) or in 1 unrelated EBS cDNA sample.

Screening for the Mutation

This mutation created a new *MboII* restriction enzyme site, which was used to screen control genomic DNA to exclude the possibility that this mutation was an innocuous polymorphism occurring in the general population. A 288 base pair fragment of PCR amplified control DNA contained one *MboII* site. Digestion of the PCR product with the *MboII* enzyme produced two fragments of 52 and 236 base pairs. The additional *MboII* restriction enzyme site created by the mutation resulted in the 288 base pair fragment being cut into four fragments of 52, 64, 172 and 236 base pairs. The digests were resolved on a 4.5% Nusieve agarose gel (figure 28). The four fragments were present in all 13 affected family members examined but only two fragments were seen in digests from one unaffected related member and 56 unrelated controls (4 EBS affected). It is therefore highly likely that the mutation underlies EBS in this family and is not a common polymorphism (χ^2 (1 d.f.)=66.27; $P<0.001$).

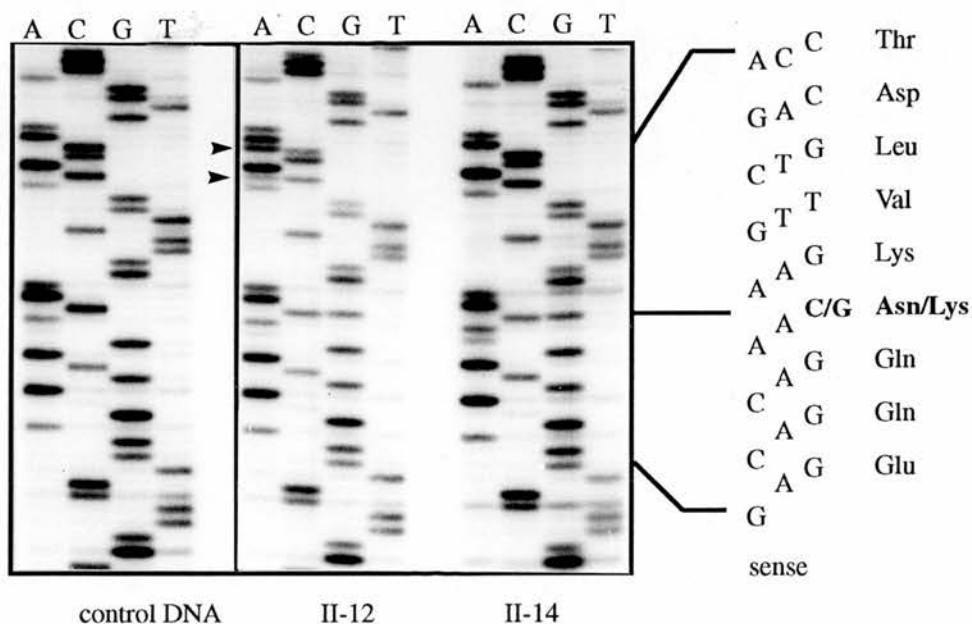


Figure 27. The DNA sequence and predicted amino acid sequence for part of the region encoding the 1A domain of keratin 5. The sequence of PCR-amplified genomic DNA from II-12, II-14 (EBS affected) of family C and from an unrelated, unaffected control. The C-to-G transition present in II-12 and II-14 results in an amino acid change from asparagine (Asn) to lysine (Lys) at codon 193. The termination bases for each lane are represented by A, C, G and T.

Two polymorphisms were present in DNA from II-12 (shown by the arrows); at codon 197, C-to-A results in an amino acid change from aspartic acid (Asp) to glutamic acid (Glu) and at codon 198, C-to-A substitution represents a silent polymorphism and does not result in an amino acid change from threonine.

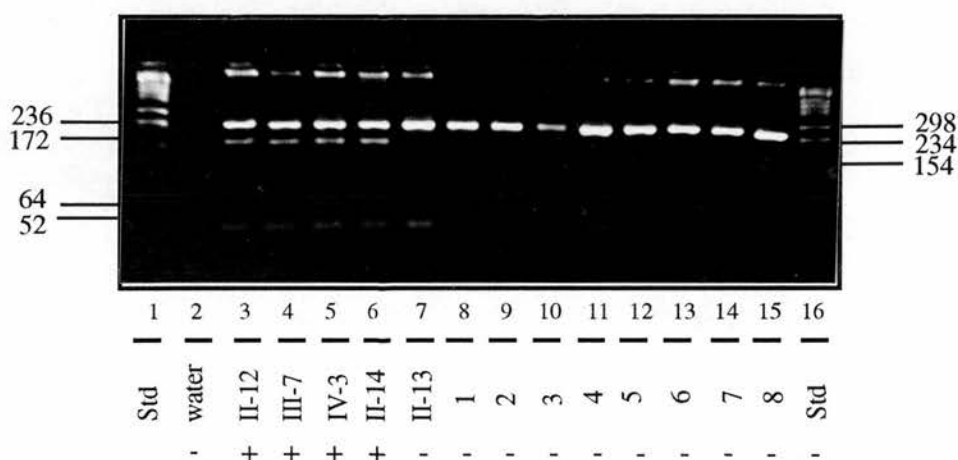


Figure 28. Ethidium bromide staining of a 4.5% Nusieve agarose gel showing analysis of genomic DNA from four affected members of family C (lanes 3-6), an unaffected member of the family (lane 7) and eight unrelated unaffected controls (lanes 8-15) for the presence of a new *MboII* site. Digestion of the 288 base pair PCR-amplified product from related but unaffected individuals or from control DNA produced two fragments of 52 and 236 base pairs. The PCR-amplified DNA from individuals affected with EBS produced four fragments after digestion with *MboII*, of 52, 64, 172 and 236 base pairs. Samples from EBS affected individuals are indicated by + and unaffected family members or controls by -. DNA molecular weight markers (std) were used to estimate the molecular weight of the fragments.

Polymorphisms in Keratin 5 and 14

No other mutations were found in any other regions of either keratin 5 or keratin 14 that were sequenced (figure 29). However sequencing data did show that this family was polymorphic for two reported polymorphisms. In keratin 5, a C-to-A base substitution at the 3rd position of codon 197, resulted in an amino acid change of aspartic acid to glutamic acid (Chan *et al.*, 1993); II-12 was heterozygous and III-5, III-14, and IV-3 were homozygous for aspartic acid. At codon 198 of keratin 5, II-12 was heterozygous for a C-to-A change; this does not result in an amino acid change from threonine and probably represents a silent polymorphism; it has also been observed in other DNA samples. In keratin 14, a T-to-C change at the 3rd position of codon 123 does not lead to an amino acid change from the predicted asparagine but represents a silent polymorphism (Coulombe *et al.*, 1991a); II-12 was homozygous for C. Codon 387 of keratin 5 was represented by serine (TCT) as reported by Lersch *et al.* (1989) and not by threonine (ACA) as by Eckert and Rorke (1988).

Genetic Linkage Analysis

Genetic linkage provided further evidence that this asparagine193 to lysine mutation is associated with the disease phenotype in this family. Analysis using the keratin 8 *Bsa*II and *Mae*II polymorphic markers on chromosome 12 were not completely informative in either linking or excluding the disease from the type II keratins (figure 30). However, non-linkage to the type I keratin, K10 *Ava*II polymorphic marker on chromosome 17, (lod score at $\theta=0.01$ was -3.92) made linkage with any type I keratin unlikely (figure 31a, b), suggesting that a type II keratin, i.e. keratin 5, was likely to be carrying the defective allele.

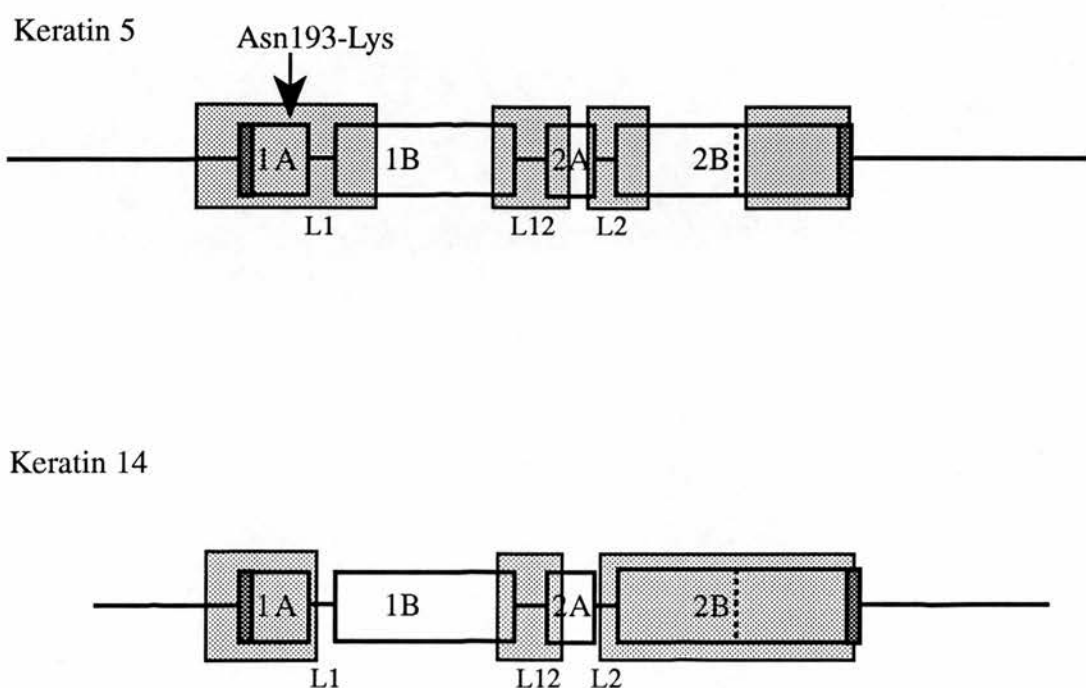


Figure 29. Diagram to show the position of the mutation identified in keratin 5 of family C. The shaded areas represent the regions of keratin 5 and 14 sequenced from this family.

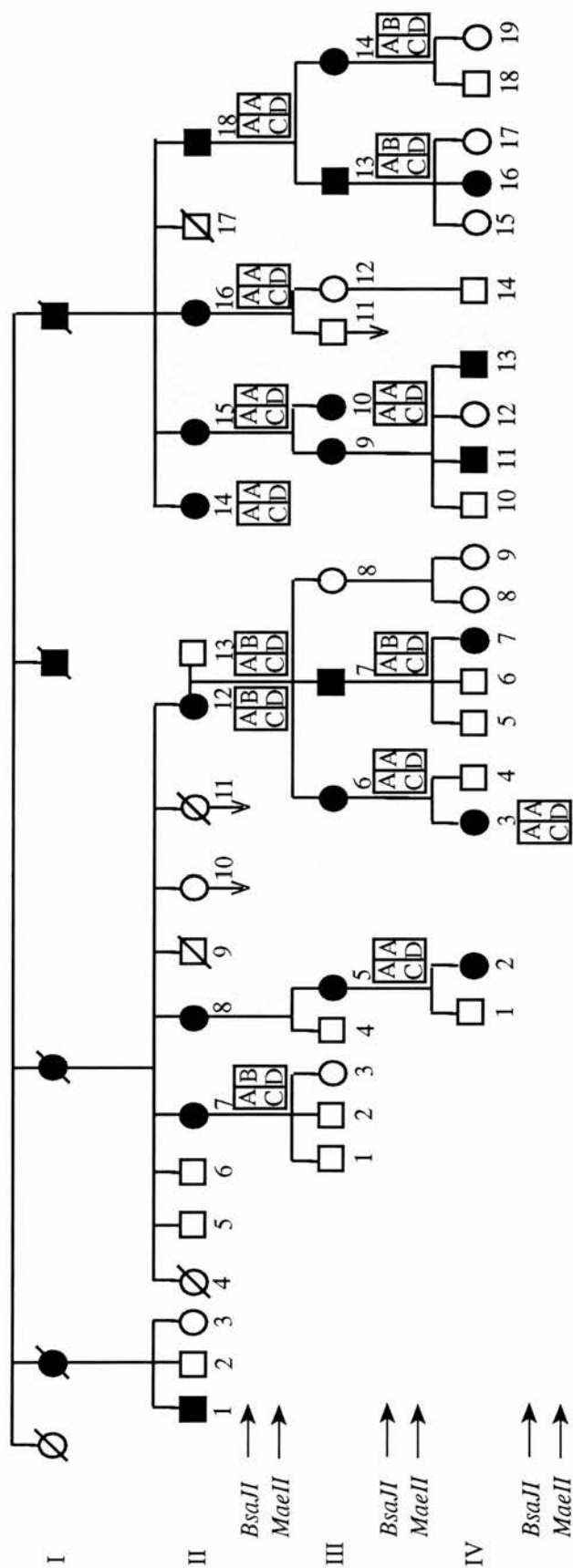


Figure 30. The figure shows analysis of the *BsaII* (alleles A and B) and *MaeII* (alleles C and D) restriction fragment length polymorphisms in keratin 8 for family C. The results for *MaeII* were uninformative in linking or excluding the disease from the type II keratins. A lod score of 1.00 when $\theta=0$ for *BsaII* suggests linkage to chromosome 12 although the result is not statistically significant.

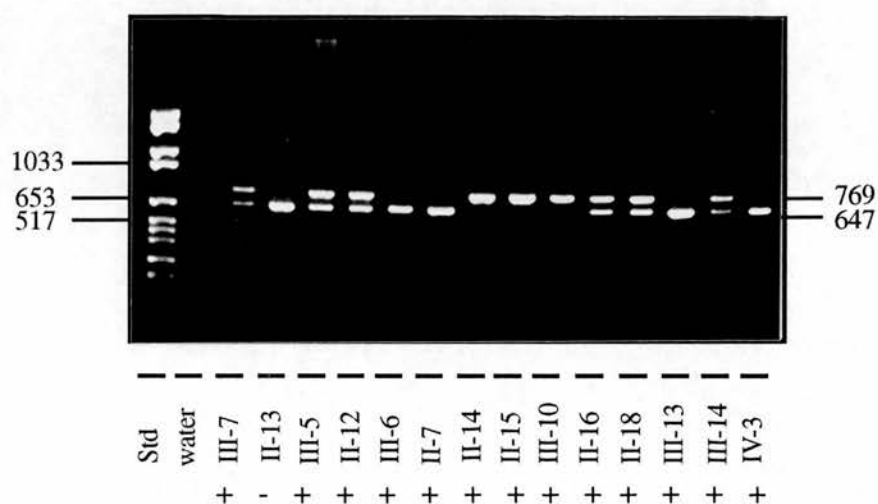


Figure 31a. Ethidium bromide staining of a 1.5% agarose gel showing digestion of the PCR amplified 769 base pair product with the *AvaII* restriction enzyme to produce fragments of 647 and 122 base pairs. These results show that EBS in family C is not linked to the keratin 10 marker (KRT-10) on chromosome 17 (see figure 31b). Samples from EBS affected individuals are indicated by + and unaffected, related controls by -. DNA molecular weight markers (std) were used to estimate the molecular weight.

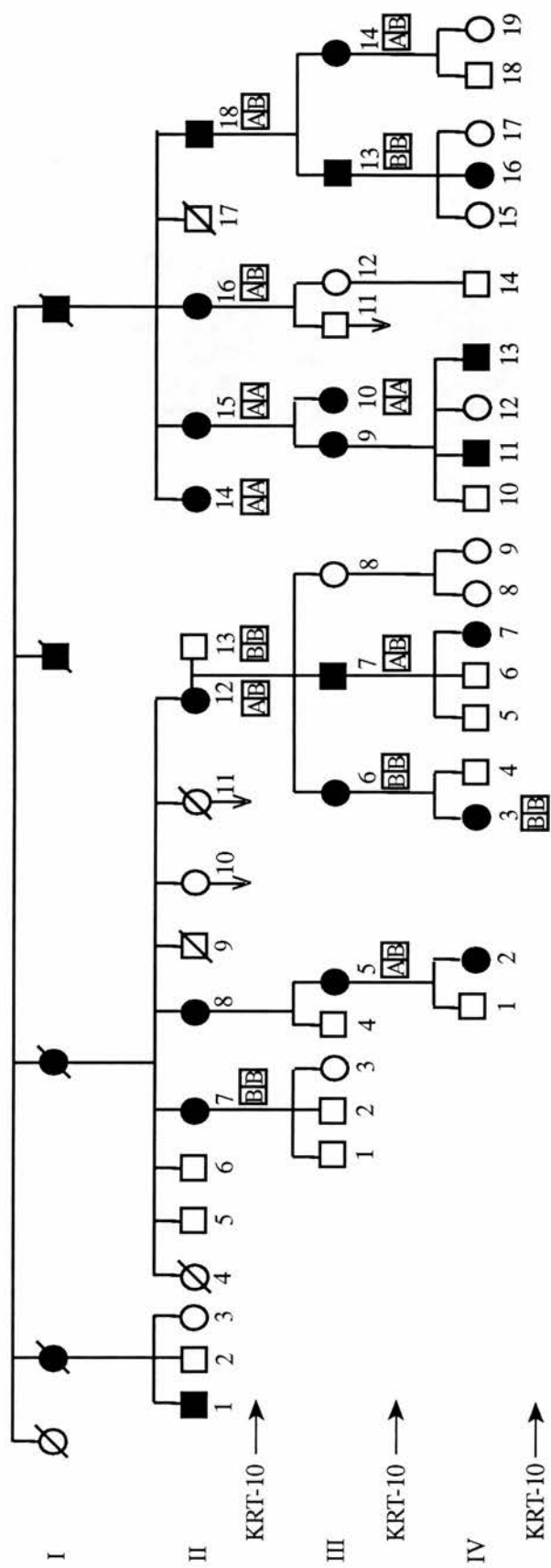


Figure 31b. The figure shows exclusion of EBS in family C to chromosome 10 marker (KRT-10) (alleles A and B). A lod score of -3.92 was obtained when $\theta=0.01$.

Discussion

Although not as highly conserved as the helix initiation peptide at the amino terminal of the α -helical 1A domain, this altered asparagine residue and neighbouring residues at the carboxy terminal of the 1A domain are relatively conserved between keratins. The helix initiation peptide is an apparent mutation “hot spot” associated with the severe form of EBS, Dowling-Meara. However, with a recent report (Hovnanian *et al.*, 1993) of a mutation in a milder form of EBS, also at the carboxy end of the 1A domain but in keratin 14, it is possible that this region could represent a cluster site for keratin mutations responsible for less severe forms of the disease. The mutation identified by Hovnanian *et al.* (1993) was slightly different though, as it was identified in a family with the rarer recessive form of EBS. However, the clinical symptoms were similar to Weber-Cockayne and blistering increased during the warmer weather.

The coiled coil arrangement of intermediate filaments is stabilised by the interactions of the two α -helical chains. In the heptad repeat of amino acids, residues in the *a* and *d* positions usually have apolar side chains and interact with those of the second chain in a knob-in-hole type of arrangement. Residues *b*, *c* and *f* are usually polar or charged and point away from the heterodimer, where they can interact with neighbouring coiled coils by ionic salt bridges or H-bonds. Residues *e* and *g* face the corresponding residues of the next keratin and form stabilising ionic interactions. The stability of a coiled coil is determined by the interactions between the α -helices which in turn depends on the particular residues in those positions. Changing a residue may alter the stability of the coiled coil, the extent of which is determined by the difference in charge, size or hydrophobicity. In intermediate filaments, residues in the *a* and *d* positions are the most highly conserved with those in the *e* and *g* positions being the next most conserved (Conway & Parry, 1988). This suggests that residues in the *a* and *d* positions are critical to maintaining the coiled coil structure, with residues at *e* and *g* providing further stability through ionic interactions. Sequence variations can be more

easily tolerated at positions *b*, *c* and *f*, although these residues are important in molecular aggregation.

The asparagine residue that is substituted by a lysine in this EBS family is at the *a* position in the heptad (figure 32). This asparagine residue is not only conserved among most type I and type II keratins but also among type III, IV, and V intermediate filaments (Conway & Parry 1988). The substitution of this residue with a lysine alters the size and also the charge, from an uncharged to a positively charged residue and is likely to perturb 10 nm filament assembly. The pitch or axial repeat per turn of the coiled coil depends on, among other factors, the apolar residues at positions *a* and *d* of the heptad (Seo & Cohen, 1993). The replacement of a charged residue in one of these critical positions could alter the pitch of that region of the keratin molecule.

Intermolecular interactions depend on the exact pitch of the coiled coil; these will be disrupted if part of the coil has become flatter or more tightly twisted.

Interesting results were reported recently by Harbury *et al.* (1993), who investigated the function of the buried hydrophobic residues at positions *a* and *d* in determining the structure of the GCN4 leucine zipper. The GCN4 leucine zipper contains a heptad sequence of $(a, b, c, d, e, f, g)_n$, similar to that of intermediate filaments, where the *a* and *d* positions are usually occupied by apolar residues. They discovered that by simultaneously altering four *a* residues and four *d* residues, the structures formed could be of two, three and four helices. This implied that these conserved residues in the GCN4 leucine zipper are important in the determining dimer rather than trimer or tetramer conformations. Substitution of asparagine16 at the *a* position with valine caused the peptide to form both dimer and trimer structures demonstrating that asparagine at position *a* is important in directing a dimer conformation.

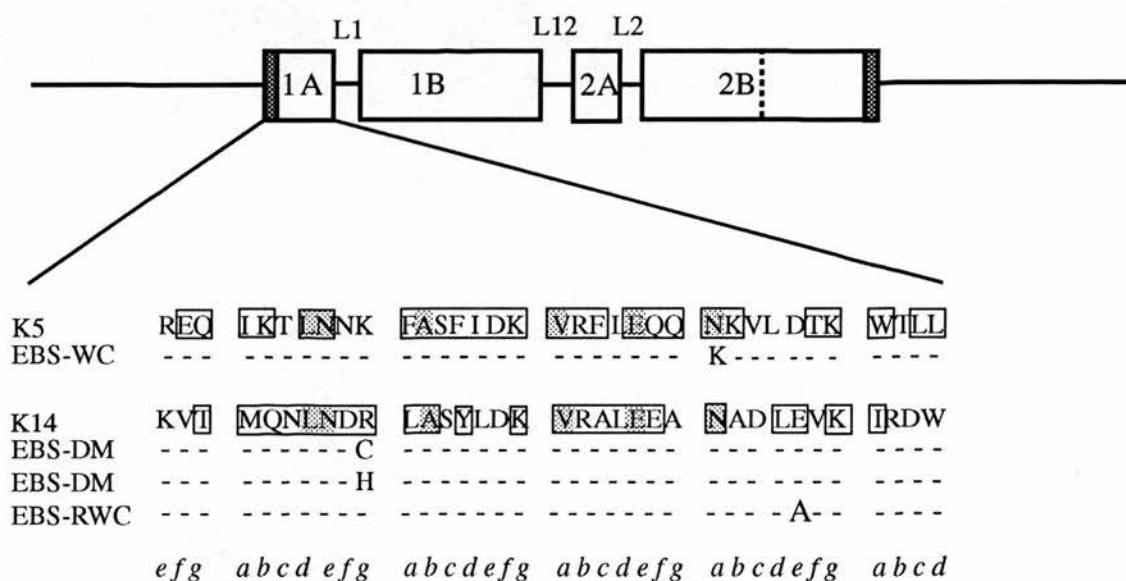


Figure 32. The amino acid sequence alignment of the α -helical 1A domain of keratin 5 and keratin 14 to show the position of the mutation in keratin 5 in family C (N-K), in relation to the mutation (E-A) in keratin 14 in a family with a recessive Weber-Cockayne like disorder (Hovnanian *et al.*, 1993). The mutations reported in EBS-DM, (Coulombe *et al.*, 1991a) in the highly conserved helix initiation peptide are shown; R-C and R-H.

The position of the amino acids in the heptad repeat are represented by *a, b, c, d, e, f, g*. Residues that are conserved within each type of keratin, type I (K14) and type II (K5), are shown by the boxes, and those residues that are identical across all types of intermediate filaments are shaded. The amino acids are shown by the single letter code.

4.4 Family D: A Point Mutation in the 2B Domain of Keratin 14

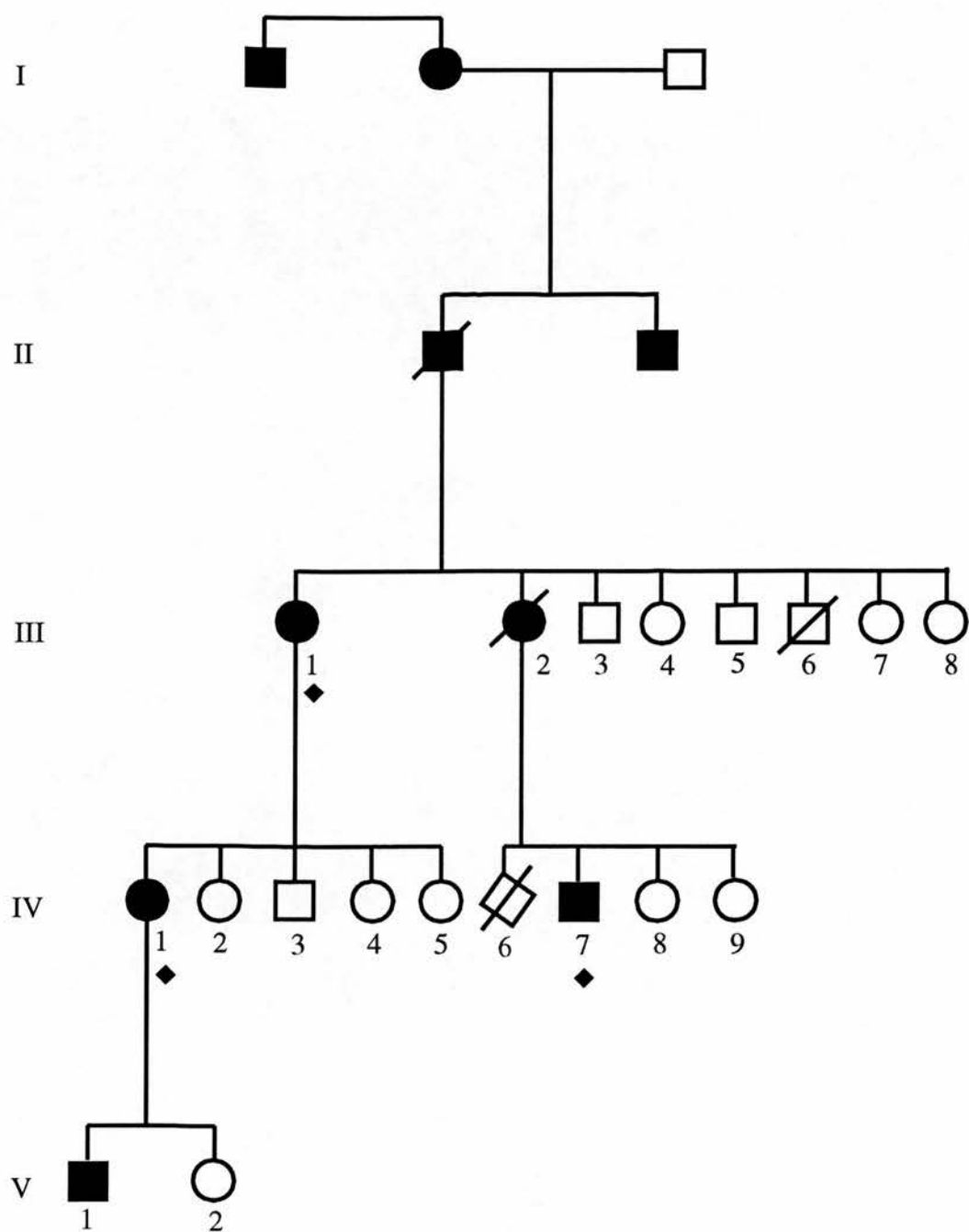
A point mutation, resulting in an arginine to cysteine amino acid change, was identified in the 2B domain of keratin 14 as the probable cause of the disease of this family.

Clinical Description

EBS Weber-Cockayne has been inherited for at least five generations of this family (figure 33). Of the four members interviewed, III-1, IV-1, IV-7 and V-1, blistering became apparent between the age of 7 months to 2 years, when learning to crawl and walk. All reported moderate to severe blistering of the feet, but were unusual in that except for IV-7, none develop blisters on their hands or at other body sites. All reported the typical Weber-Cockayne seasonal variation, with more severe blistering in the summer. IV-7 has suffered from blisters on his hands only since leaving school and obtaining a job that involves the regular use of screwdrivers. His blisters are much less seasonal than previously, suggesting that the continual friction from using tools at work is responsible for inducing blisters on his hands.

Identification of a Mutation in the 2B Domain

DNA sequencing of PCR amplified genomic DNA from two affected family members (III-1 and IV-1) revealed a C-to-T nucleotide substitution at the first position of codon 388 in the 2B domain of keratin 14. This resulted in an amino acid change from arginine to cysteine (figure 34). This same mutation has been identified in a further two EBS-WC families (Dr. Rugg, personal communication). There were no other sequence variations in the regions of either keratin K5 or K14 that were sequenced (figure 35) although several polymorphisms were detected. IV-1 was heterozygous for the polymorphisms in keratin 14, at codons 94 (alanine to threonine) and 123 (asparagine,



◆ indicates patients from whom blood samples were obtained

Figure 33. Pedigree of family D

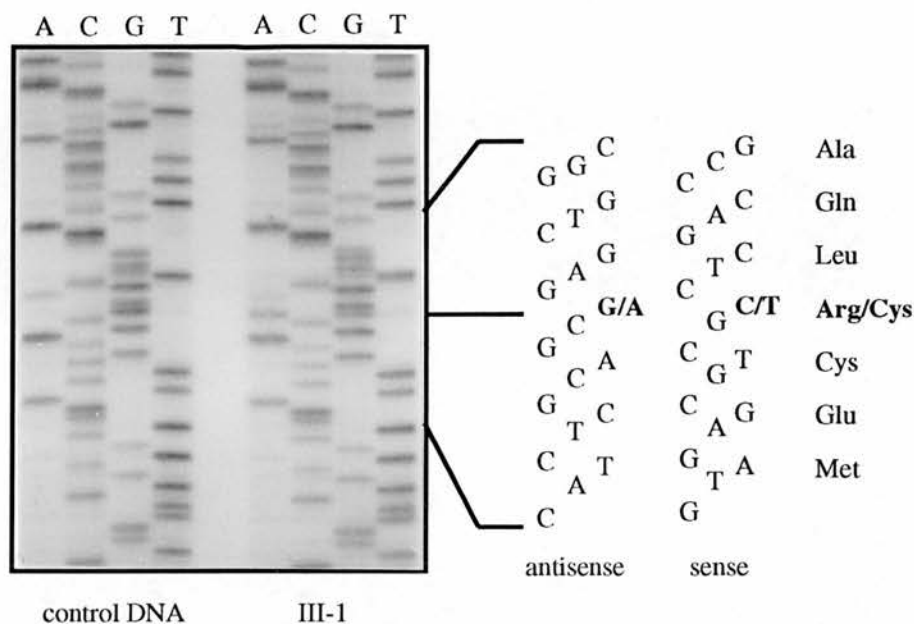
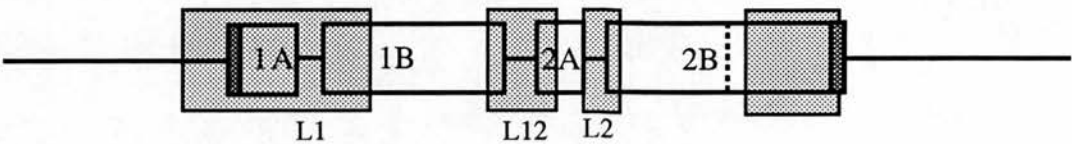


Figure 34. The DNA sequence and predicted amino acid sequence for part of the region encoding the 2B domain of keratin 14. The sequence of PCR-amplified keratin 14 genomic DNA from III-1 (EBS affected) of family D and from an unrelated unaffected control. An amino acid change from the predicted arginine (Arg) to cysteine (Cys) at codon 388 results from the C-to-T substitution present in III-1. The termination bases in each lane are indicated by A, C, G and T.

Keratin 5



Keratin 14

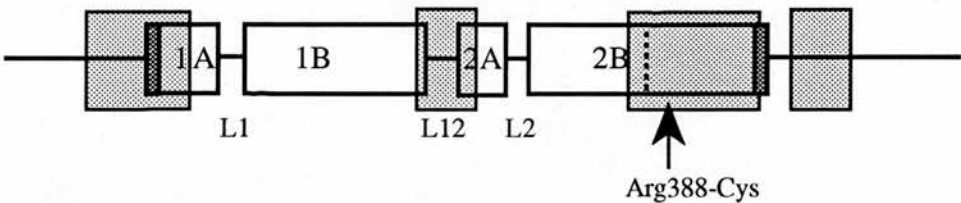


Figure 35. Diagram to show the position of the mutation identified in keratin 14 of family D. The shaded areas represent the regions of keratin 5 and keratin 14 sequenced from this family.

a silent polymorphism), and in keratin 5 at codon 197 (aspartic acid to glutamic acid) III-1 was homozygous for aspartic acid.

Screening for the Mutation

This mutation did not create a restriction enzyme site and to screen for the mutation several different approaches were carried out. One method involved designing a primer which, in conjunction with the base change, created a new *PstI* restriction enzyme site. Only samples containing the mutation and therefore this new site were expected to be cut by *PstI*. However, after PCR amplification, digestion of the DNA fragments with *PstI* resulted in apparent digestion of both control and EBS affected genomic DNA, although two control cDNA samples were not cut. This method was repeated using different primers to first amplify a larger fragment of DNA and then this fragment was re-amplified with internal primers to create a *PstI* site. This time only the EBS samples and not the six control genomic DNA samples were cut.

One explanation to the above discrepancies was that first time the wrong keratin had been amplified. It was possible that a pseudogene of keratin 14 or keratin 17, which has a high sequence homology to keratin 14, had been amplified. Comparison of the keratin 14 and keratin 17 sequences (Raychaudhury *et al.*, 1986) indicated that if keratin 17 had been amplified the fragment would have been approximately 200 base pairs larger as intron V of keratin 17 (that was within the fragment amplified) is much larger than that of keratin 14. However, the sequence of a keratin 14 pseudogene shows 95% sequence identity with the coding region and 93% sequence identity with the intron sequences of the functional keratin 14 (Savtchenko *et al.*, 1988a). In addition, in the pseudogene the arginine residue at codon 388 has been substituted for cysteine making it questionable as to whether the mutation identified in these EBS-WC families was “real”. Comparison of the primer sequences made it highly probable that the pseudogene had been amplified when all the control samples were cut with *PstI*.

However, since some of these control samples when amplified and sequenced with different primers showed no mutation, whereas samples from the EBS patients did, this indicated that the same mutation as in the pseudogene had occurred in the functional keratin 14 resulting in EBS in these families. Other approaches to screen the control DNA were investigated. Another keratin 14 primer was designed within an intron but only poor amplification was achieved under the chosen conditions.

An alternative approach using PASA analysis was investigated. This technique is very sensitive to PCR conditions. Although, cDNA from one patient (from one of the other families with this same mutation) did amplify with both the wild type and mutant primers, and cDNA from TR146 cells was amplified only with the wild type primer, successful amplification of control genomic DNA was not achieved with either primer under the conditions chosen.

This region of the 2B domain of keratin 14 was also sequenced from 2 unrelated EBS cDNA samples, and 1 unrelated genomic DNA EBS sample. A further 14 unrelated control genomic DNA samples have been sequenced (by Dr. Rugg, personal communication). No mutation at codon 388 has been identified in any of these samples, indicating that this mutation is probably the cause of EBS in these three families but a reliable screen or DNA sequencing of more control samples is required to confirm this.

Discussion

The number of control samples screened or sequenced so far, do not statistically confirm that this mutation is causative for EBS in family D, but no other sequence variations (apart from some common polymorphisms) were found in the other regions of keratin 5 and keratin 14 that were sequenced (figure 35). The fact that this residue is altered in the keratin 14 pseudogene identifies it as a residue sensitive to mutation.

Although no cDNA (from cultured keratinocytes) was obtained from family D, in one of the other two EBS-WC families with this mutation, cysteine in place of arginine has been identified in the cDNA (Dr. Rugg, personal communication). This suggests that although this substitution occurs in the keratin 14 pseudogene when this same substitution occurs in the functional keratin 14 and is translated into functional proteins (unlike pseudogenes) it is disruptive to the keratin cytoskeleton and can result in the EBS phenotype.

This putative mutation in family D is just four amino acids away from a leucine to proline mutation at codon 384 identified in a family with generalised blistering of EBS-K (Bonifas *et al.*, 1991b). A possible explanation for the difference in severity of the disease between these two families is the position of the substituted residues within the heptad repeat. The arginine residue that is substituted for a cysteine in family D is at the *e* position and the leucine to proline mutation is at the *a* position of the heptad repeat in the α -helical 2B domain (figure 36). Since residues at the *a* and *d* positions are the most highly conserved with those in the *e* and *g* positions the next most conserved (Conway & Parry, 1988) it is predicted that residues in the *a* and *d* positions are the most critical to maintaining the structure of the coiled coil. Substitution of an amino acid in either of these positions (*a* or *d*) might be expected to result in a more severe phenotype than substitution of a residue at an *e* or *g* position. Residues at the *e* and *g* positions face those of the corresponding keratin and form stabilising interactions. The replacement of arginine by cysteine at the *e* position of the heptad in family D may be disruptive due to the difference in charge and size between the two residues and, also results in two adjacent cysteine residues which could potentially form a disulphide bond.

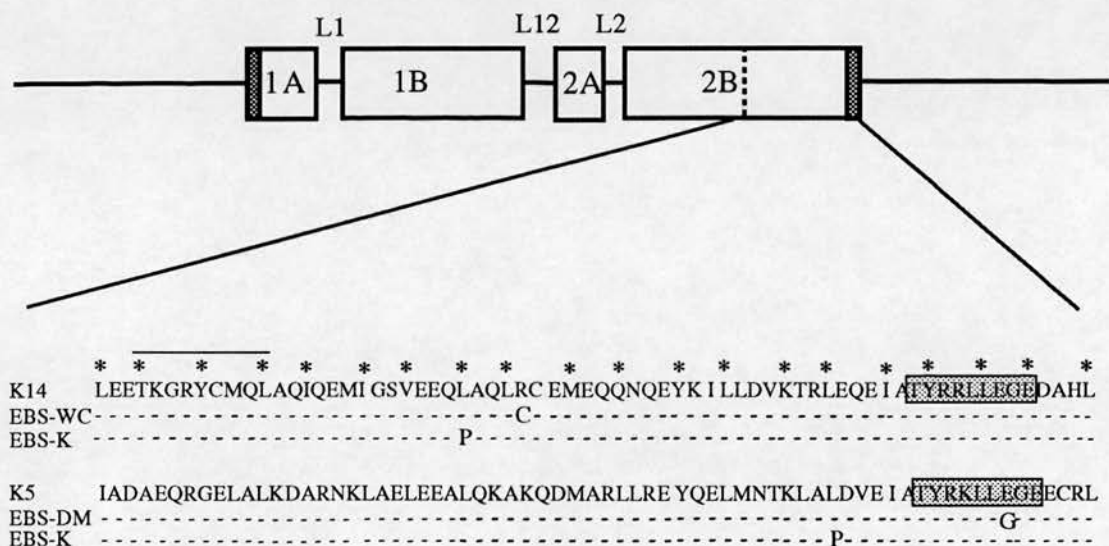


Figure 36. The amino acid sequence alignment for part of the α -helical 2B domain of keratin 5 and keratin 14 to show the position of the mutation in family D (EBS-WC) in keratin 14 (R-C) in relation to the mutation (L-P) reported by Bonifas *et al.* (1991b) in an EBS-K family. The amino acids are represented by the single letter code. The shaded residues represent the highly conserved sequence motif, at the carboxy terminal of the rod domain, that is common to all known intermediate filaments. Other mutations reported in the 2B domain of keratin 5 include the E-G mutation in the highly conserved motif in an EBS-DM family (Lane *et al.*, 1992), and a L-P mutation in an EBS-K family (Dong *et al.*, 1993).

The hydrophobic residues (*a* and *d*) of the heptad repeats are shown by *, the stutter, near the centre of the 2B domain, is due to a reversal in polarity which causes a break in the heptad repeat and is marked by (—).

A point to consider is whether by comparison of the pseudogene and the functional keratin 14 gene sequences other residues likely to cause EBS could be identified. Residues that have been mutated in the pseudogene are susceptible to mutation and if, in the functional keratin 14 gene this same residue is altered it might be detrimental to the integrity of the cytoskeleton resulting in a disease phenotype.

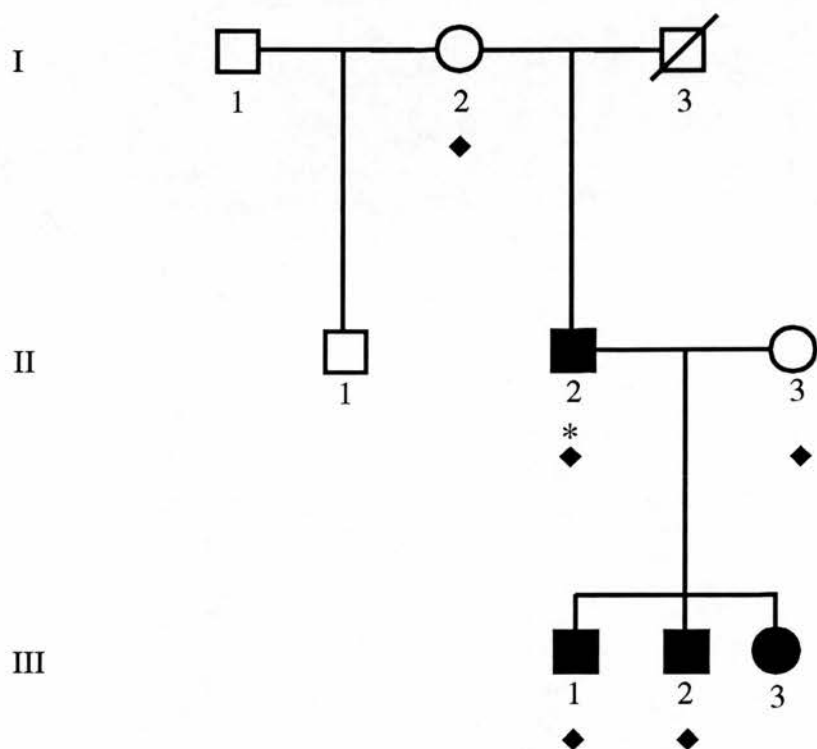
4.5 Family E : No Mutation Identified in Keratin 5 or Keratin 14

Although extensive DNA sequencing of both the keratin 5 and keratin 14 genes was carried out, no mutation was identified in this family.

Clinical Description

Blistering in this family arose in the second generation shown on the pedigree (figure 37). The disorder is relatively severe, particularly in the youngest generation.

Blistering developed during the first two years of life and in common with Weber-Cockayne, blisters are predominantly on the hands and feet. However, there are also oral blisters, sometimes on the tongue, which makes eating of certain foods such as crisps difficult or impossible. Although, in the literature, oral blisters are not usually reported for Weber-Cockayne, preliminary data collected for the UK National EB Register (see Appendix IV) indicate that they may be more common than previously thought. Nail dystrophy is also present in some members of this family. The youngest generation (III-1, III-2 and III-3) aged between 8 and 13 years old also develop blisters at other sites subjected to friction, such as the waistline, neck, and the thighs from riding a bicycle; they also suffer from anal fissures. There is also the marked seasonal variation typical of Weber-Cockayne. The transition from primary to secondary school has aggravated the disease due to the increased amount of walking required between lessons compared to at primary school. EBS can be as much a social problem as a disease: children, especially, do not like to be considered different from their friends and are often prepared to suffer from blisters so that they can join in activities such as football and dancing.



- ◆ indicates patients from whom blood samples were obtained
- * indicates the patient from whom a skin biopsy was obtained

Figure 37. Pedigree of family E

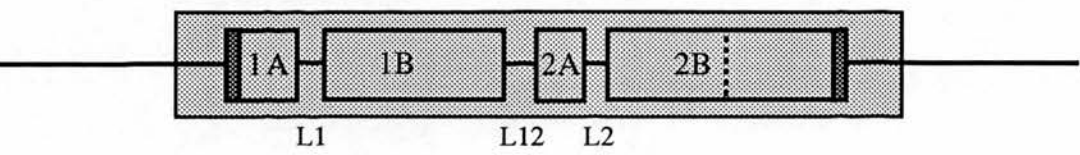
DNA Sequencing

Keratinocytes were cultured from a skin biopsy obtained from II-2 and cDNA extracted for DNA sequencing. Sequencing of the target regions of both keratin 5 and keratin 14, regions where other EBS-WC mutations have been identified, did not reveal any sequencing variations. Further regions of both keratin 5 and 14 were sequenced (figure 38); in keratin 5 codon 261 (glutamic acid) and codon 271 (glutamic acid) were as reported by Eckert & Rorke (1988) and not by Lersch *et al.* (1989), and codon 387 (serine) as reported by Lersch *et al.* and not Eckert & Rorke, but no further sequence discrepancies were identified. Regions containing known polymorphisms were sequenced from genomic DNA when blood samples were obtained from other members of the family (II-2, III-1 and III-2 EBS affected, I-2 and II-3 unaffected). This data identified the family as being polymorphic for some of these nucleotide substitutions (see genetic linkage analysis). II-2, III-1 and III-2 were all homozygous for glycine at codon 138 in keratin 5 which represents the K5b allele.

Genetic Linkage Analysis

Genomic DNA was extracted from blood samples obtained from three affected (II-2, III-1 and III-2) and from two unaffected (I-2 and II-3) family members. Genetic linkage analysis was carried out using several polymorphic markers. With the D17S800 probe, on chromosome 17, the disease co-segregated with the marker suggesting a defect in a type I keratin (K14) (figure 39). DNA sequencing of known polymorphisms in keratin 14 and keratin 5 supported this. The presence of the polymorphisms at codons 77, 94 and 123, in keratin 14 (figure 40) indicated that the disease was linked to the type I keratins. The polymorphism at codon 197 in keratin 5 showed non-linkage of the disease to type II keratins with a lod score of -4.7 when $\theta=0$ (figure 41). Analysis using the keratin 10 *AvaII* and the keratin 8 *BsaII* and *MaeII* polymorphic markers were uninformative in either linking or excluding the disease from type I or type II keratins (figure 42, 43).

Keratin 5



Keratin 14

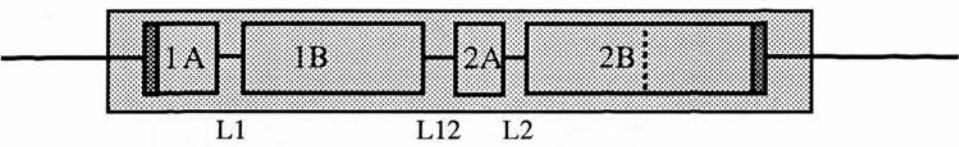


Figure 38. Diagram to show the regions of keratin 5 and keratin 14 sequenced from family E, represented by the shaded areas.

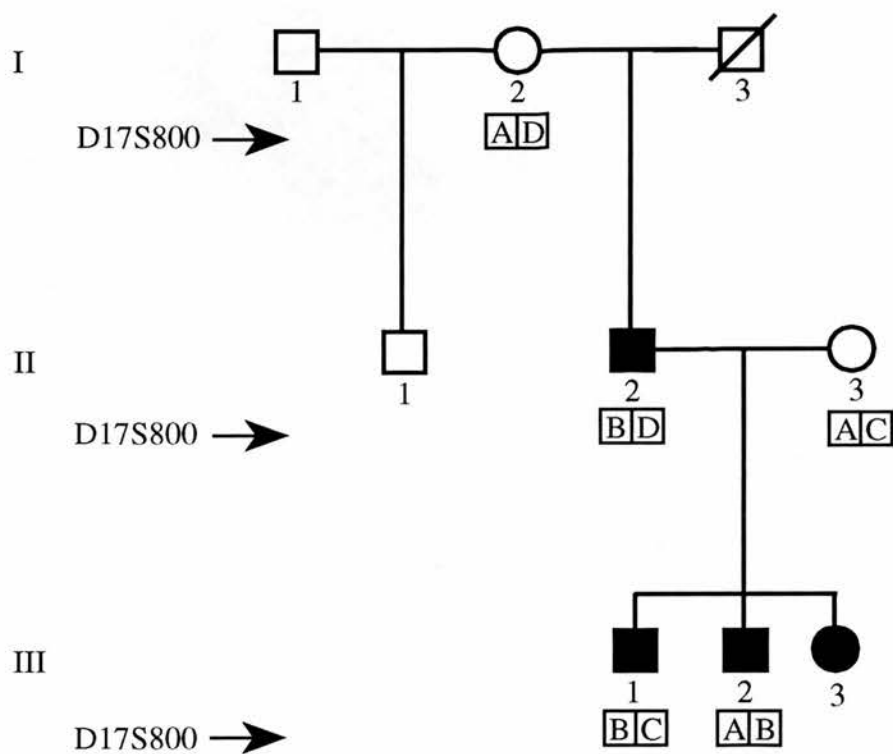


Figure 39. The figure shows co-segregation of the disease in family E with the polymorphic marker D17S800 on chromosome 17 (alleles A, B, C and D), indicating a possible defect in a type I keratin. However, the lod score at $\theta=0$ was 0.3 and is not statistically significant.

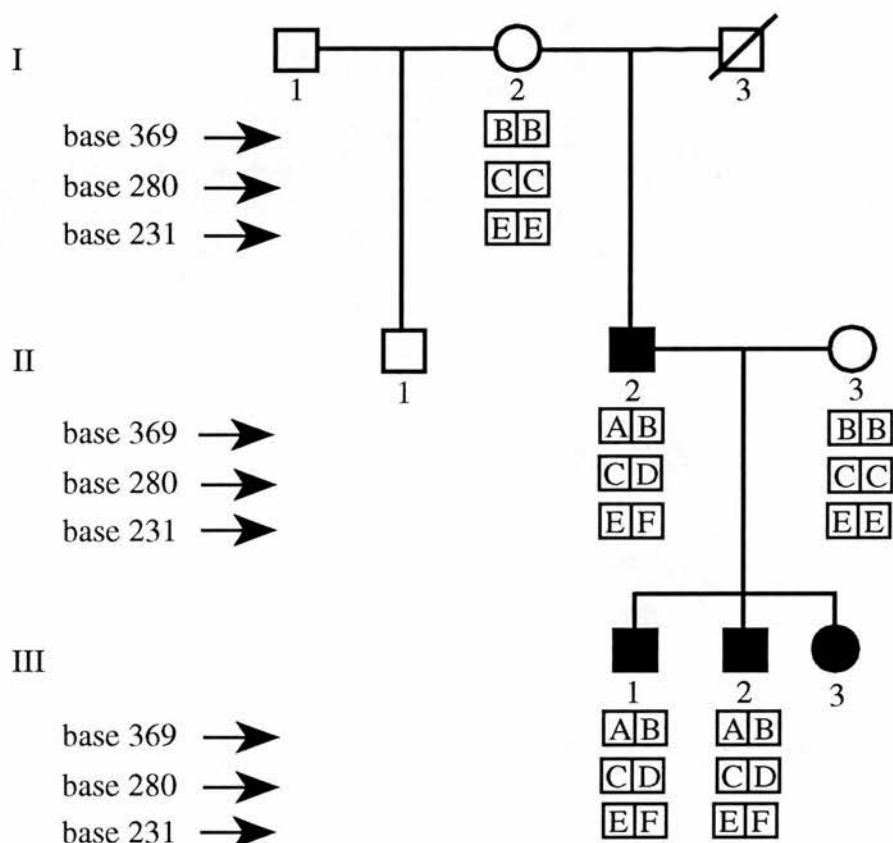


Figure 40. The figure shows linkage of the disease in family E to type I keratins using polymorphisms in keratin 14 as polymorphic markers. A T-to-C transition at nucleotide 369 (alleles A and B) represents a silent polymorphism with no amino acid change from asparagine at codon 123. At nucleotide 280 a G-to-A substitution results in an amino acid change from alanine to threonine at codon 94 (alleles C and D). A C-to-T substitution at nucleotide 231 (alleles E and F) represents another silent polymorphism with no change from the serine residue at codon 77. The lod scores for all three markers were not high enough to be statistically significant, at $\theta=0$ the lod score for each was 0.3.

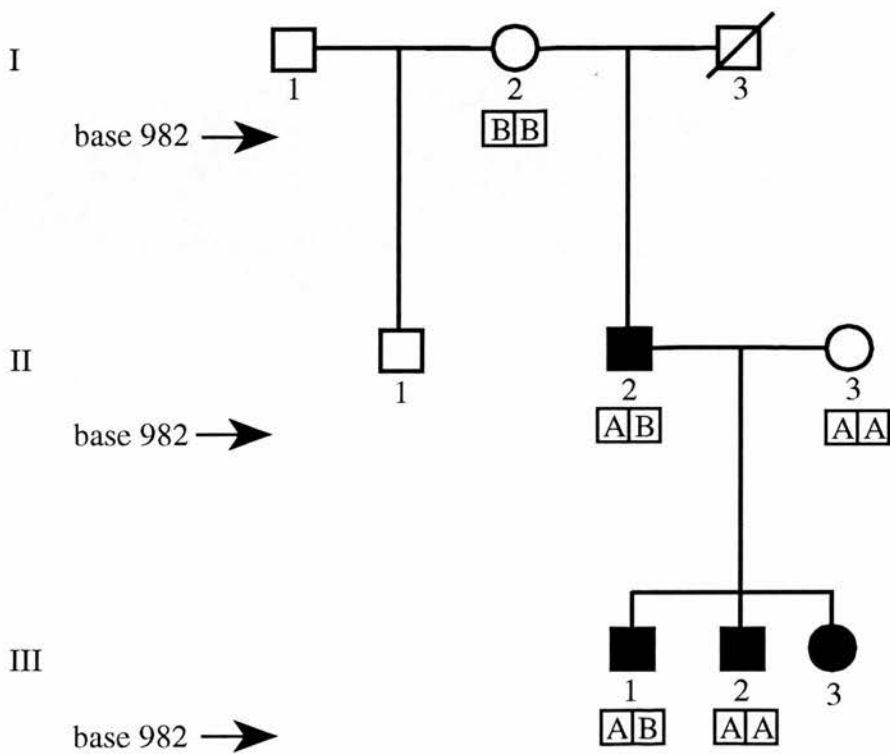


Figure 41. The figure shows non-linkage of the disease in family E to type II keratins using the polymorphism at nucleotide 982 in keratin 5 as a polymorphic marker (alleles A and B). The C-to-A transition results in an amino acid change at codon 197 from aspartic acid to glutamic acid. A lod score of -4.70 when $\theta=0$ is significant to exclude the genetic defect in this family from chromosome 12.

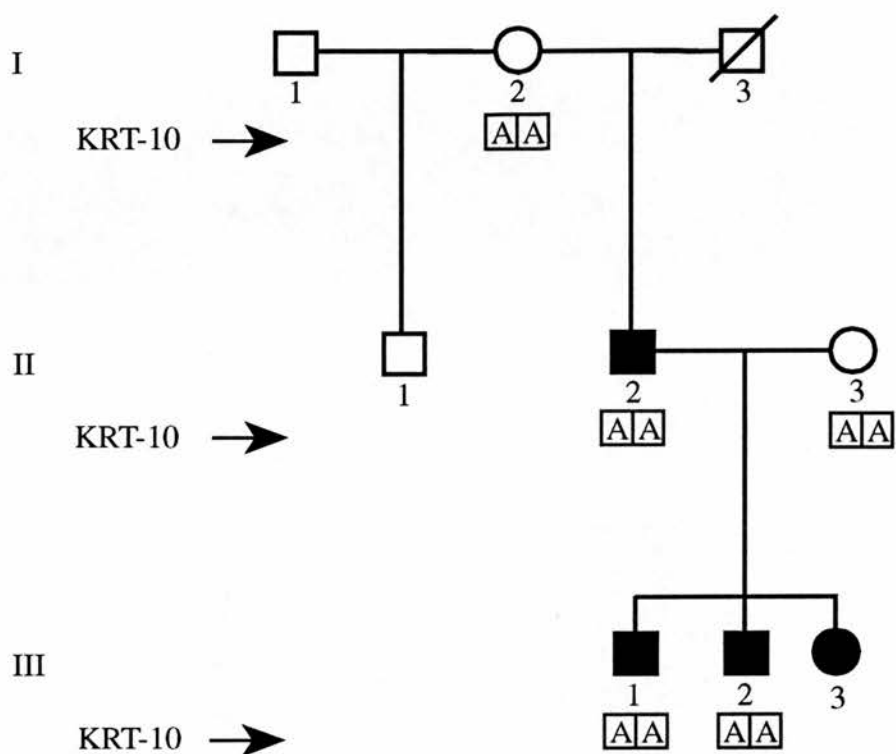


Figure 42. The figure shows genetic analysis with the keratin 10 marker (KRT-10) on chromosome 17, alleles A and B: the results were not informative in linking or excluding the disease in family E from type I keratins.

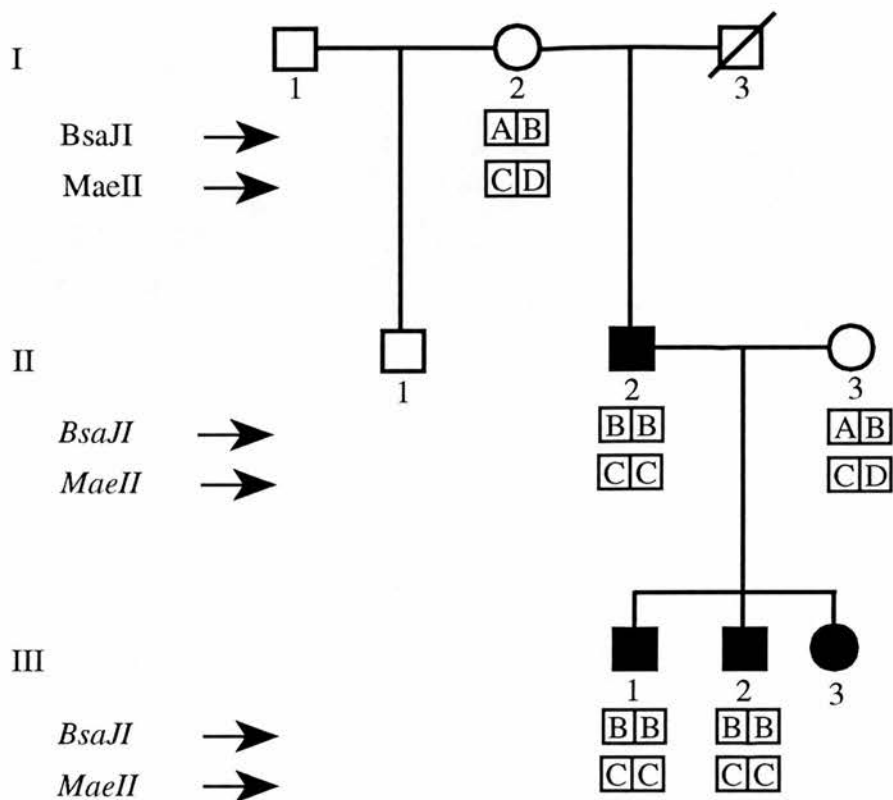


Figure 43. The figure shows genetic analysis using the K8 restriction fragment length polymorphic markers. The *Bsa*JI site is represented by alleles A and B, and the *Mae*II site by alleles C and D, but from these results the disease in family E could not be linked or excluded from the type II keratins.

Re-examination of the sequencing data of keratin 14 did not lead to identification of any sequence variation.

Expression of Keratins by Immunoblotting

Cytokeratins were extracted from cultured keratinocytes from II-2 and analysed for keratin expression by SDS polyacrylamide gel electrophoresis (10% acrylamide gels) and immunoblotting with anti-keratin antibodies. The keratins were detected by staining the immunoblots with the following antibodies: AE3 to type II keratins, AE1 to type I keratins, LLOO1 to K14, RCK102 to K5 and K8, E3 to K17, α IFA to most intermediate filaments and India ink that stains all proteins (figure 44a, b). Staining with these antibodies did not reveal any difference in migration or deficiency in the keratins expressed between the EBS keratinocytes and TR146 cells (TR146 also express K8, K18 and, K17 instead of K16). The predominant keratins expressed were K5, K6, K14 and K16, in approximately equal amounts. Small amounts of K1 and K10 were detectable but there was no reactivity with antibodies to K8 (LE41), K18 (LE61) or K19 (LP2K).

Figure 44. Immunoblots of cytoskeletal extracts stained with anti-keratin antibodies to show the pattern of keratins expressed by a primary keratinocyte culture from II-2 (affected by EBS) of family E and from TR146 cells (as a control). Prestained molecular weight markers (std) were used to estimate the molecular weight: phosphorylase B = 106 kD; bovine serum albumin = 80 kD; ovalbumin = 49.5 kD; carbonic anhydrase = 32.5 kD.

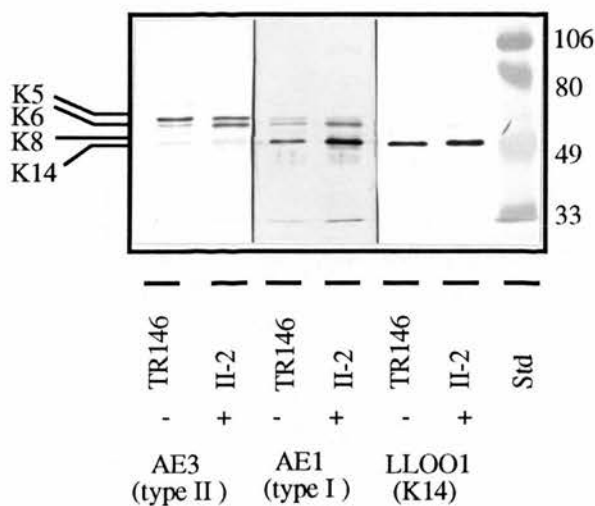


Figure 44a. Staining with AE3, which reacts with all type II keratins, identified K5 and K6 as the predominant type II keratins expressed by both cultures. II-2 was homozygous for the K5b allele, represented by the single band for K5. TR146 cells, in addition to K5 and K6, expressed small amounts of K8.

Stained with AE1, to all type I keratins, showed K14 to be the major type I keratin expressed.

With LLOO1, a monoclonal antibody specific to K14, II-2 showed a normal staining pattern.

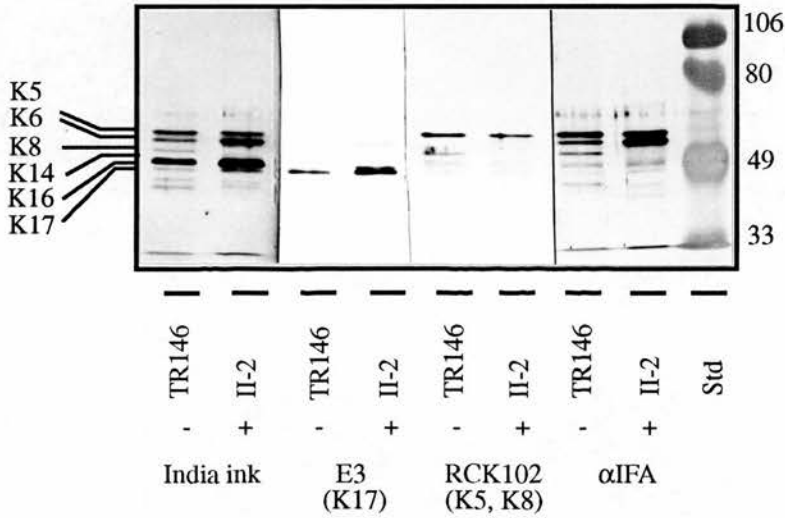


Figure 44b. Immunoblot stained with India ink identified K5, K6, K14 as the predominant keratins expressed by the cells, with smaller amounts of K16 present (K17 in TR146 cells).

Stained with E3, a monoclonal antibody specific to K17 (some K16 staining in the EBS cells).

With RCK102 to K5 and K8, the EBS cells (II-2) showed normal staining of K5 with, and as expected, no K8 staining. TR146 cells stained for K8 as well as K5.

With αIFA, which reacts with all intermediate filaments, a stronger reactivity to the type II keratins (K5 and K6) than to the type I keratins (K14 and K16) was observed.

Discussion

These findings raise the question as to whether all EBS cases are due to keratin 5 or keratin 14 defects. Further DNA sequencing of the head and tail domains of keratin 5 and in particular keratin 14, in view of the linkage data, is necessary to completely exclude an alteration in the primary sequence of these keratins. Even then, one of these keratins could still be abnormal. An abnormality affecting a higher level of keratin filament assembly might result in the same clinical phenotype as a single amino acid substitution in the primary sequence. Alternatively, a mutation in a minor keratin in the basal cells might have a similar effect in reducing the integrity of these cells. The probable linkage of the disease in this family to type I keratins means other possible candidates are keratin 15 which is expressed along with keratin 5 and 14 in basal epidermal cells or, keratin 9, which is specifically expressed in the palms and soles. It is possible that a defect in a keratin associated protein, such as those involved in insertion of intermediate filaments into desmosomes or hemidesmosomes, would also be disruptive to the functioning of the keratin cytoskeleton.

CHAPTER 5

DISCUSSION

All subtypes of EBS share the common feature of intraepidermal blistering of the skin, in the sub-nuclear region of the basal keratinocytes, in response to mechanical trauma. Prior to the commencement of this study, the underlying pathogenetic mechanism was unknown, but during the course of this project, the genetic defect was identified in several affected families (Bonifas *et al.*, 1991b; Coulombe *et al.*, 1991a; Lane *et al.*, 1992). Mutations in the genes encoding the basal keratins, K5 and K14, have now been identified as being responsible for some, if not all cases of EBS-DM, EBS-K and EBS-WC. These are the predominant keratins in the basal keratinocytes and mutations in these keratins would be expected to affect primarily keratin filaments of the basal rather than spinous layers.

A structural protein defect was proposed as the cause of EBS after abnormal clumping of tonofilaments (which could be labelled specifically with antibodies to keratins K5 and K14) was observed in the keratinocytes of EBS-DM (Anton-Lamprecht *et al.*, 1979; Niemi *et al.*, 1983; Tidman *et al.*, 1988; Ishida-Yamamoto *et al.*, 1991).

However, the absence of an observable abnormality of the tonofilaments in EBS-WC made it uncertain as to whether this form of EBS was also the result of a keratin defect (Pearson, 1971; Hanke & Anton-Lamprecht, 1982). Nonetheless, it appeared far more likely that EBS-WC was a disorder of keratin filaments than the result of a defect in enzyme package or production. Therefore, soon after the work for this thesis was commenced, it was decided to concentrate on the keratin cytoskeleton in EBS-WC.

5.1 Different Approaches that Led to the Same Conclusion

Studies in several independent laboratories, using different techniques, established that keratin gene mutations were the underlying cause of EBS in some families. The role of transgenic animals (Vasser *et al.*, 1991) and analysis of patient material (Bonifas *et al.*, 1991b; Coulombe *et al.*, 1991a; Lane *et al.*, 1992), in predicting and confirming this statement is discussed below. My project, along with other simultaneous studies, have

identified keratin gene mutations in further EBS families. Collectively, these investigations demonstrate that mutations in either the keratin 5 or keratin 14 genes, can produce a range of EBS clinical phenotypes, Dowling-Meara, Koebner and Weber-Cockayne.

5.1.1 Transgenic Animal Studies

With their interest in the function of keratin filaments, Fuchs *et al.*, having established the effects of keratin mutants in an *in vitro* system (Albers & Fuchs, 1987, 1989; Coulombe *et al.*, 1990; see Chapter 1, 1.16), continued their investigations by studying the effect of such keratin mutants on the keratin filament network in a living organism. They created transgenic mice by inserting mutant keratin 14 genes, with 135 amino acids deleted from the carboxy terminal end (lacking all of the non-helical carboxy tail and more than 30% of the central α -helical domain), into the mouse embryos (Vassar *et al.*, 1991). The resultant mice developed intraepidermal blistering at birth or after mild mechanical trauma, and other stratified squamous epithelia containing keratin 14, such as the oral mucosa and tongue also blistered. Ultrastructural examination of the skin, and cultured keratinocytes from these mice, showed clumping of tonofilaments, almost identical to that observed in the Dowling-Meara variant of EBS. There was a high neonatal mortality and the phenotype was considered to be more severe than EBS-DM. Transgenic mice that lacked only 50 amino acids from the carboxy terminal, a mutant known to produce only mild perturbations in filament assembly *in vitro*, did not show any blistering or basal cell cytolysis, although there was a mild degree of aggregation of keratin between the tonofilaments. These two phenotypes appeared to flank the human disease EBS but indicated that keratin defects could be responsible for EBS-DM. A series of keratin 14 mutants were then created (Coulombe *et al.*, 1991b) that produced a range of EBS-like phenotypes in transgenic mice; the greater the number of amino acids deleted from the carboxy terminal the more severe the phenotype. The severity of the phenotype in the mice corresponded to the effect of the mutation on keratin filaments in

cultured keratinocytes. Mutants that only moderately disrupted filament assembly produced a much milder disease phenotype, corresponding to the human EBS-K or EBS-WC. In the milder phenotypes, blisters were located only on the paws, areas subjected to greatest friction. Basal cell cytolysis occurred but there was no clumping of the tonofilaments, although the filaments were shorter and more disorganised than those of wild type (Coulombe *et al.*, 1991b). The variation in phenotype of these transgenic mice, created by different mutations in the same gene, raised the possibility that different EBS variants could be caused by mutations in the same gene, with the severity dependant on the particular residue that was altered and the role of the residue in the keratin filament network.

5.1.2 Analysis of Patient Samples

The above reverse genetics approach of correlating the effect of an altered gene in transgenic mice to a human disease phenotype identified the probable cause of EBS and, increased our understanding of the structure and function of keratins. However, conclusive proof that mutations in the keratin 14 gene (or keratin 5) were responsible for EBS required analysis of material from patients. Concurrently, several research groups analysed material from EBS patients and confirmed this. Patient samples (blood samples or cultured keratinocytes from skin biopsies) were analysed in a number of different ways; genetic linkage analysis, immunoblotting followed by staining with monoclonal antibodies and DNA sequencing. Specific keratin gene mutations were identified in several EBS families.

Genetic Linkage Analysis

Genetic linkage analysis was used to predict which chromosome carried the abnormal gene responsible for EBS. As EBS was proposed to be caused by an abnormality of the genes encoding either keratin 5 (on chromosome 12) or keratin 14 (on chromosome 17), DNA polymorphic markers within or near these putative defective genes were

used. Analysis of several families by this approach indicated that keratin gene abnormalities were the underlying cause of the Koebner and Weber-Cockayne variants of EBS. Two families were analysed by Bonifas *et al.* (1991a). In one, affected by EBS-WC, the disease was linked to chromosome 12 (type II keratins) and in the second family, affected by EBS-K the disease was linked to chromosome 17 (type I keratins). Subsequent linkage analysis studies provided further evidence that the Koebner and Weber-Cockayne phenotypes were linked to abnormalities on chromosome 12 and chromosome 17, most probably keratin 5 and keratin 14 respectively. Ryynanen *et al.* (1991b) mapped EBS in one family to chromosome 12 and McKenna *et al.* (1992a) reported linkage data for two EBS-WC families. In one family, where blistering was confined to the soles, the disease was linked to chromosome 12 and in the second family, where blistering affected the hands and feet, linkage was to chromosome 17. Hoyheim *et al.* (1992) mapped EBS-WC in one family to chromosome 12.

Immunoblotting with Anti-Keratin Antibodies

A different approach, taken by Lane *et al* (1992) detected a keratin 5 abnormality in a family affected with EBS-DM. Monoclonal antibodies were used to analyse keratin expression by cultured keratinocytes and this identified the region of the keratin molecule that was abnormal. Keratins were extracted from cultured keratinocytes from several members of the family and were resolved by SDS polyacrylamide gel electrophoresis, transferred onto nitrocellulose paper by Western blotting and reacted with anti-keratin antibodies. When immunoblots were stained with monoclonal antibodies to keratin 5 some members of the family were heterozygous for the K5a/K5b polymorphism, identified by the staining of a doublet band due to the different electrophoretic mobilities of the two alleles. This allowed the reaction of both keratin 5 alleles to be individually observed. When immunoblots were then stained with the monoclonal antibody α IFA, one of the keratin 5 bands failed to stain in some affected

individuals. Since the antibody α IFA reacts with the helix termination peptide of all intermediate filaments, this lack of staining indicated that there was a defect in this domain of keratin 5.

DNA Sequencing

Further analysis, by DNA sequencing, of some of the above families led to the identification of specific keratin gene mutations. A point mutation, in the α -helical 2B domain of keratin 14, was identified in the EBS-K family which had shown linkage of the disease to chromosome 17 (Bonifas *et al.*, 1991b). DNA sequencing of keratin 5 cDNA, from members of the EBS-DM family with abnormal keratin 5 staining on immunoblots, identified a mutation at codon 475 (glutamic acid to glycine), in the highly conserved helix termination peptide (Lane *et al.*, 1992). Coulombe *et al.* (1991a), examined the coding sequence of keratin 14 from cDNA extracted from cultured keratinocytes from two unrelated EBS-DM families. In both, point mutations were identified in keratin 14 and at the same amino acid residue, arginine 125. However, different nucleotide substitutions in the codon were involved, resulting in a different amino acid change (C-to-T, arginine to cysteine; G-to-A, arginine to histidine). This arginine residue, highly conserved throughout evolution, is in the helix initiation peptide of the α -helical rod domain of the keratin molecule, a region already shown by deletion experiments *in vitro*, to be critical for intermediate filament assembly.

The mutations described above were only present in affected members of the family and not in unaffected members or in unrelated controls, thereby excluding these mutations as common polymorphisms occurring within the general population. These studies illustrated that same clinical phenotype, Dowling-Meara, could result from mutations in different regions of the keratin molecule (helix initiation peptide or helix termination peptide) and in different members of the heterodimer (keratin 5 or keratin 14). They

also confirmed that different clinical phenotypes, EBS-DM and EBS-K could be caused by mutations within the same gene, as predicted by the transgenic mice studies (Coulombe *et al.*, 1991b).

5.2 Keratin Gene Mutations Identified in This Study

My project investigated, in parallel with the above studies, the possibility that mutations in the genes encoding either keratin 5 or keratin 14 could underlie the relatively mild Weber-Cockayne variant of EBS. Five EBS-WC pedigrees were studied. Skin biopsies were obtained for keratinocyte culture; cDNA and cytokeratins were subsequently extracted and, genomic DNA was extracted from blood samples. Using a combination of the above three techniques, DNA sequencing, genetic linkage analysis and immunoblotting with monoclonal antibodies, point mutations were identified in four of the families.

The mutations were identified in either the keratin 5 or keratin 14 gene and were all within the central rod domain (figure 45a). In one, family C, the mutation was in the α -helical 1A domain of keratin 5 (asparagine193 to lysine) but carboxy terminal to the highly conserved helix initiation peptide, and in another (family D) a tentative mutation was found in the α -helical 2B domain of keratin 14 (arginine388 to cysteine). The remaining two families (A and B) both had mutations in the non-helical central L12 linker domain, one in keratin 5 (arginine331 to cysteine) and the other in keratin 14 (valine270 to methionine).

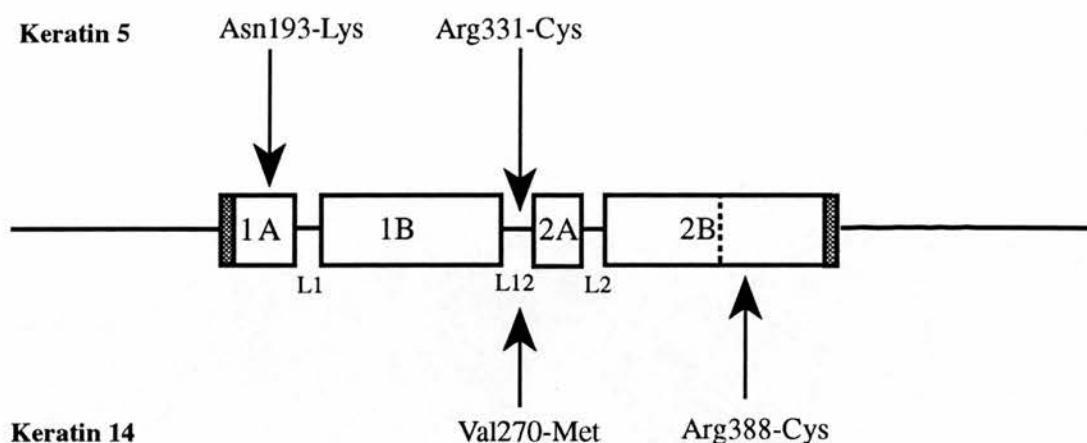


Figure 45a. Schematic diagram to show the position of the EBS-WC mutations identified in this study. The shaded areas represent the highly conserved helix initiation and helix termination peptides.

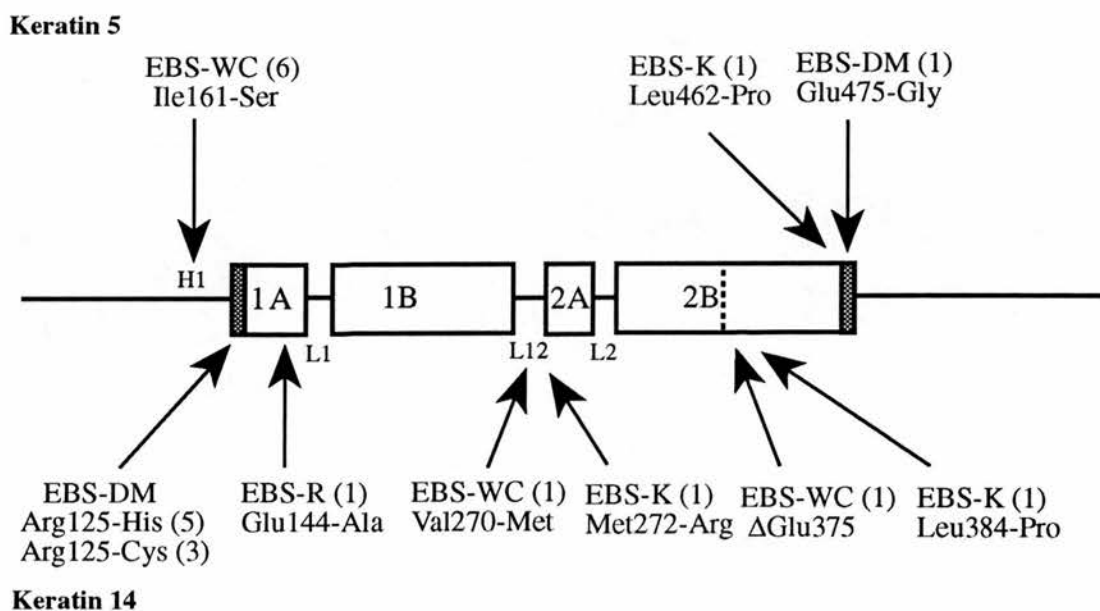


Figure 45b. Schematic diagram to show the position of the other reported mutations in EBS-DM, EBS-K, EBS-WC and recessive EBS (EBS-R). The figures in brackets represent the number of unrelated cases with the same mutation.

Cytokeratin extracts, from cultured keratinocytes from affected members of families A, B and E, showed no variation in the migration rate or pattern of keratin staining compared to controls, when analysed by SDS polyacrylamide gel electrophoresis and immunoblotting. All the families had typical Weber-Cockayne EBS and although there was some variation in severity between different members of the same family and between families, there were no major differences to suggest why the mutations occurred at different positions of the keratin molecule.

5.3 Clustering of Pathological Mutations

There are now several further reports of keratin gene mutations in EBS-K and EBS-WC some of which have already been mentioned in Chapter 4. Although the total number of mutations identified in the three variants of EBS, Dowling-Meara, Koebner and Weber-Cockayne, is still relatively small, the emerging trend would suggest that the location of the mutation in the keratin molecule, rather than in which member of the heterodimer, keratin 5 or keratin 14, the mutation occurs, determines the severity of the disease phenotype. All the mutations identified as causing the most severe variant of EBS, Dowling-Meara, are at the extreme highly conserved ends of the rod domain (in the helix initiation peptide and the helix termination peptide), regions shown by deletion mutagenesis experiments to be critical for filament assembly *in vitro*. Mutations found in the Koebner and Weber-Cockayne variants are internal to, or outside these terminal ends of the rod domain, with the exception of one EBS-K mutation that has recently been identified in the helix termination peptide of keratin 5 (Dong *et al.*, 1993). For these two variants of EBS, the mutations appear to be clustering at certain points; the H1 domain, the L12 linker domain, the 1A domain and the centre of the 2B domain. How accurate the correlation is between the position of the mutation and the severity of the disease phenotype will only become apparent as the number of identified mutations increases.

Reported keratin mutations responsible for EBS-K and EBS-WC are summarised in figure 45b, and include a leucine to proline substitution at codon 384 in the 2B domain of keratin 14 in EBS-K (Bonifas *et al.*, 1991b). In another EBS-K family, early investigations had provided tentative linkage to genetic markers on chromosome 1q (Humphries *et al.*, 1990), but a mutation was later identified in the L12 linker domain of keratin 14, methionine272 to arginine (Humphries *et al.*, 1993). Linkage of EBS-K to chromosome 12 in another family (Ryynanen *et al.*, 1991b) led to the identification of a mutation in keratin 5 of a leucine to proline amino acid change at codon 462 in the highly conserved helix termination peptide (Dong *et al.*, 1993). This family had previously been reported to have an enzyme deficiency in galactosyl hydroxylysyl glucosyltransferase (Savolainen *et al.*, 1981). However these deficiencies, unlike the leucine to proline mutation, did not consistently co-segregate with the disease phenotype, indicating that this keratin mutation rather than the enzyme abnormality was the primary cause of blistering in this family. Two EBS-WC families were reported to have the same nucleotide substitution resulting in an isoleucine to serine mutation at codon 161 in the H1 region of the non-helical head domain of keratin 5 (Chan *et al.*, 1993). This same nucleotide substitution has also been identified in four unrelated British families (Rugg *et al.*, 1993b).

There is one report of a keratin point mutation in a family with a recessive EBS-WC like disorder which results in a glutamic acid to alanine substitution at codon 144 in the α -helical 1A domain of keratin 14 (Hovnanian *et al.*, 1993). Unlike the dominantly inherited cases of EBS where the mutations are heterozygous with both the normal and the mutant nucleotide present, in this family the affected offspring were homozygous for the single nucleotide substitution while both parents and unaffected offspring were heterozygous. Recently a three base pair deletion has been detected in another EBS-WC family (Chen *et al.*, 1993) leading to the deletion of glutamic acid at codon 375 in

the α -helical 2B domain of keratin 14. This is the first demonstration that not all keratin defects causing EBS are point mutations.

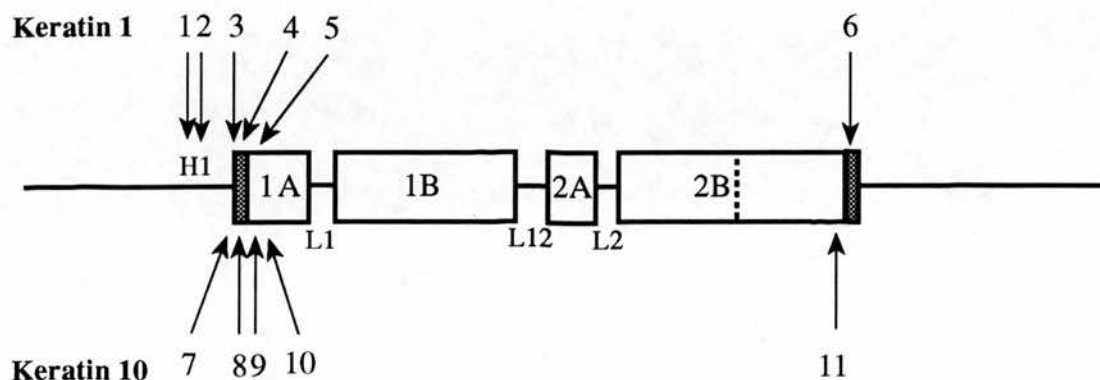
The majority of the mutations identified as causing EBS-DM are in the same codon of the highly conserved helix initiation peptide (figure 44). Following the identification of mutations in keratin 14 at codon 125 in the two EBS-DM families studied by Coulombe *et al.* (1991a), a further study of ten EBS-DM families (Stephens *et al.*, 1993) revealed that five had mutations at this same residue. In four, the same nucleotide substitution resulted in a histidine in place of arginine and in the fifth, arginine was replaced by cysteine. The remaining families were examined for mutations at codon 475 in the helix initiation peptide of keratin 5, where a mutation in another EBS-DM family had been identified (Lane *et al.*, 1992). However, no mutations were found suggesting that unlike codon 125, this residue is not a mutation hot spot for EBS-DM; mutations have not yet been reported in these five remaining EBS-DM families.

Keratin Gene Mutations in BCIE

When discussing the location, disease severity and the effect of these mutations on the functioning of the cytoskeleton it is of interest to compare them with the recently identified keratin gene mutations in bullous congenital ichthyosiform erythroderma (BCIE), a disorder characterised by lysis in the supra-basal layers of the epidermis, where keratin 1 and keratin 10 are the predominant keratins. A common feature of both EBS and BCIE is increased cell fragility, of the basal cells in EBS and the suprabasal cells in BCIE. The suprabasal keratinocytes of BCIE also show abnormal clumping of tonofilaments, a characteristic of the basal keratinocytes of EBS-DM. An abnormality in either keratin K1 or K10 was proposed as the cause of BCIE. A similar reverse genetics approach as for EBS was applied. Transgenic mice containing a keratin 10 mutation were constructed and these mice produced a disease phenotype similar to BCIE (Fuchs *et al.*, 1992). Point mutations in either keratin 1 or keratin 10 have now

been identified, by several independent laboratories, in families affected with BCIE (Cheng *et al.*, 1992; Chipev *et al.*, 1992; Rothnagel *et al.*, 1992, 1993, 1994; Chipev *et al.*, 1994; McLean *et al.*, 1994, Yang *et al.*, 1994). A similar picture to EBS is emerging for BCIE, with mutations clustering to certain regions of the keratin molecule. The majority of those identified are from relatively severe cases with the mutations located in the highly conserved extreme ends of the rod domain; other mutations have been identified in the H1 domain of the non-helical head and in the α -helical 2B domain (figure 46).

These findings demonstrate that, similar to keratins K5 and K14, the K1 and K10 keratins provide mechanical stability to the cells. When the filament network is compromised in some way, such as a sequence alteration, the cells become more susceptible to mechanical shearing forces, resulting in cell lysis.



Keratin 1	residue	number of affected families	
1. H1	Val154-Gly	1	Yang <i>et al</i> , 1994
2. H1	Leu160-Pro	1	Chipev <i>et al</i> , 1992
3. 1A	Ser185-Pro	1	McLean <i>et al</i> , 1994
4. 1A	Asn187-Ser	2	McLean <i>et al</i> , 1994
			Yang <i>et al</i> , 1994
5. 1A	Ser192-Pro	1	Yang <i>et al</i> , 1994
6. 2B	Glu489-Gln	1	Rothnagel <i>et al</i> , 1992
Keratin 10			
7. 1A	Asn154-His	1	Chipev <i>et al</i> , 1994
8. 1A	Arg156-His	7	Cheng <i>et al</i> , 1992
			Rothnagel <i>et al</i> , 1992, 1993
			Chipev <i>et al</i> , 1994
1A	Arg156-Cys	2	Chipev <i>et al</i> , 1994
			Rothnagel <i>et al</i> , 1993
1A	Arg156-Ser	1	McLean <i>et al</i> , 1994
1A	Arg156-Leu	1	Rothnagel <i>et al</i> , 1993
1A	Arg156-Pro	1	McLean <i>et al</i> , 1994
9. 1A	Tyr160-Asp	1	Chipev <i>et al</i> , 1994
1A	Tyr160-Asn	1	Rothnagel <i>et al</i> , 1994
10. 1A	Leu161-Ser	1	Rothnagel <i>et al</i> , 1992
11. 2B	Leu443-Gln	1	Chipev <i>et al</i> , 1994

Figure 46. Schematic diagram to show the position of BCIE mutations identified in keratins K1 and K10. The shaded areas represent the highly conserved helix initiation and helix termination peptides.

5.4 Keratin Gene Mutations Suggest a Structural Role for Keratins

Collectively, these EBS and BCIE studies demonstrate that keratin gene mutations can be disruptive to the functioning of the cell cytoskeleton and have provided the first real evidence that some, if not all intermediate filaments, play an important structural role. Although all intermediate filaments share a similar secondary structure, they differ in their cell and tissue specificity and may differ in any structural function; some cells may be more sensitive or more prone than others to mechanical stress. Various studies, including a comparison of the viscoelastic properties of all three cytoplasmic network systems, microtubules, microfilaments and intermediate filaments, have provided evidence to support a structural role for intermediate filaments. Using rheologic methods, Janmey *et al.* (1991) concluded that vimentin (type III) intermediate filaments are flexible at low strain, but harden at high strain, resisting breakages under stresses which would cause actin microfilament networks to rupture. Keratin intermediate filaments have not yet been examined by this method and they may differ from vimentin in their tolerance of mechanical trauma. Nuclear lamin intermediate filaments are thought to provide a structural function, since nuclear envelopes, when formed in the absence of nuclear lamins, are fragile and lyse on physical trauma (Newport *et al.*, 1990). Over expression of a type II hair keratin in cortical hair cells of transgenic mice resulted in abnormal keratin filament organisation which led to brittleness and hair breakage (Powell & Rogers, 1990). This was suggested to be due to aggregation of the type II keratin due to absence of the type I which then interfered with normal integrity of the network. The epidermal keratin filaments are also very stable proteins with strong protein-protein interactions between the heterodimers. They are one of the few proteins that survive the process of terminal differentiation (keratinisation); most other cytoplasmic proteins and organelles are lost during this transition to form a relatively impermeable epidermis. Hair keratins are even more stable than epidermal keratins due to the presence of cysteine-rich sequences (Fuchs, 1991).

However, the results of other investigations still question the precise function of these ubiquitous intermediate filament proteins. It has been demonstrated that, at least in a few cultured cell lines, intermediate filaments are not essential for cell growth. A rat hepatoma cell line (Venetianer *et al.*, 1983) and a human adrenal cortex carcinoma-derived cell line (Hedberg & Chen, 1986) are both lacking detectable intermediate filament expression but, can grow and divide normally with no major alterations in cellular organisation or morphology. Mouse myeloma cells (MCP-11 cell line) lack intermediate filaments but vimentin synthesis can be induced after treatment with phorbol ester 12-O-tetradecanolyphorbol-13-acetate, this synthesis is likely to be regulated at the transcriptional level (Giese & Traub, 1986). However, although intermediate filaments may not be essential for all cell culture lines, they may have a structural function when present in differentiated tissues. Baribault and Oshima (1991) used gene targeting to introduce null and subtle mutations into the germ line of mice. When both keratin 8 alleles in the mouse embryonic stem cells were disrupted the cells could still differentiate in tissue culture into normal looking embryoid bodies with an apparently normal epithelia. No intermediate filaments (type I keratins K18, K19, or vimentin) were detected in these cells, indicating that keratin 8 and its partner are apparently not needed for this *in vitro* program of embryonic development. Disruption of the intermediate filament networks by microinjection of anti-intermediate filament antibodies (Klymkowsky *et al.*, 1983) or the expression of mutated intermediate filaments did not prevent the cells from maintaining their shape or undergoing mitosis and movement, but it is not known how long these cultured cells can survive (Albers & Fuchs, 1987, 1989; Gill *et al.*, 1990).

5.5 The Role of Individual Residues

The identification of mutations within the same gene that can produce a disease phenotype of variable severity has raised many questions regarding the role of individual residues to the overall integrity of the cell cytoskeleton. Various factors may

determine the effect of a particular amino acid change on the overall assembly and functioning of the keratin filament network, and the consequent clinical phenotype. Substitution of an amino acid may alter the size, charge or α -helicity of the monomer leading to varying degrees of distortion when this molecule interacts with the other member of the pair to form a dimer and then at later stages of intermediate filament assembly. Mutations may, however, differ in the level of structure at which they influence filament assembly and therefore be detrimental for different reasons. They may interfere with the molecular recognition necessary for dimer formation, or in the formation of tetramers or protein folding. Mutations affecting the early stages of filament assembly would be expected to have the most severe effects. The deletion of an amino acid, as in one EBS-WC family (Chen *et al.*, 1993), is predicted to lead to the misalignment of dimers due to changed positions of subsequent amino acid side chains around the axis of the helix rather than to disruption of the monomer helix as caused by some point mutations. Incorrect dimer formation would affect the assembly of tetramers and higher order structures and therefore the overall cytoskeleton. The position of the mutation with respect to disruption of the network was demonstrated experimentally by Letai *et al.* (1992). Proline substitutions internal to the rod domain and within a long stretch of coiled coil were better tolerated than those at the extreme ends of the rod domain of keratin 14. In some cases a mutation may create a potential new phosphorylation site which could affect the stability of the network.

Most deletion and point mutagenesis studies of keratins, except for those by Letai *et al.* (1992), have been confined to the non-helical head and tail or to the end domains of the rod and so there are very little data to predict the effect of mutations involving the internal part of the keratin molecule. Two EBS-K mutations have led to the introduction of a proline residue into the rod domain. In one, a leucine to proline mutation at codon 384 in the centre of the 2B domain of keratin 14 (Bonifas *et al.*, 1991b) is predicted to decrease the α -helical conformation and likely to introduce a kink

in this region of the monomer causing distortion to the subsequent formation of dimers (Hoffman, 1991). In the other, a leucine to proline substitution in the highly conserved carboxy end of the rod domain of keratin 5 (Dong *et al.*, 1993) is predicted, from the findings of Letai *et al.*, to be more disruptive to the filament network. However, there was apparently no major difference in the clinical phenotype of the two pedigrees of EBS-K: both were associated with generalised blistering of moderate severity.

The contribution of individual residues to the overall structure of the filament network, and the resulting clinical severity of the mutations is emphasised by comparing two mutations in the L12 linker domain of keratin 14; the valine270 to methionine mutation described here in family B and the methionine to arginine mutation at codon 272 identified by Humphries *et al.* (1993). Although within two residues of each other, the mutation at codon 270 produced a relatively mild phenotype with blistering predominantly of the feet and hands, compared to the mutation at codon 272 which was associated with a generalised blistering tendency. It should, however, be remembered that the clinical distinction between EBS-WC and EBS-K is often not clearly defined, particularly when cases are reported by different clinicians.

The mutations in the H1 domain of the type II keratins in both EBS and BCIE are of a slightly different nature to the others as they create potential new phosphorylation sites. Deletion mutagenesis studies by Wilson *et al.* (1992) have already shown the non-helical head domain of keratin 5, and maybe that of other type II keratins, to be important in filament assembly *in vitro*. The isoleucine161 to serine mutation in keratin 5 (Chan *et al.*, 1993; Rugg *et al.*, 1993b) creates a new potential phosphorylation site, serine-glutamine-arginine, for protein kinase C (Woodgett *et al.*, 1986). In keratin 1 the leucine160 to proline mutation converts the adjacent serine residue into a potential phosphorylation site by the p34 *cdc2* kinase (Chou *et al.*, 1990; Peter *et al.*, 1991). The phosphorylation state, particularly of the non-helical head domain, is involved in

the regulation of intermediate filament assembly and disassembly. Phosphorylation at either of these extra sites could destabilize the filament network in a similar way to that of lamin A and vimentin intermediate filament networks (Chou *et al.*, 1990).

One residue, arginine 125 in the helix initiation peptide of K14, and the corresponding residue in K10, arginine 156, has been identified as a mutation hot spot responsible for EBS-DM and BCIE respectively. This residue is highly conserved throughout intermediate filaments, present as arginine or lysine in 50 of 51 published sequences (arginine in 47 out of 51, and three of the remaining four are lysine with same charge as arginine; Cheng *et al.*, 1992). It is a CpG site which represents a hot spot for mutation by methylation and deamination of cytosine (C) to thymidine (T). More than 30% of point mutations causing human genetic diseases occur within CpG dinucleotides, with over 90% of these being C-to-T transitions or G-to-A on the complementary strand (Cooper & Youssoufian, 1988). There are other CpG dinucleotides within the rod domain, several of which are in the highly conserved terminal ends. As yet, no mutations in either EBS or BCIE have been identified at them. Letai *et al.* (1993) engineered seven of these C-to-T transitions in keratin 14 and examined the effects on keratin filament network formation when the mutants were transfected into cultured epidermal cells (SCC-13 cells) and, on their ability to form filaments *in vitro*. All these mutations were less detrimental to the filament network than the EBS /BCIE arginine mutation. Mutations at these CpG sites might therefore, cause more subtle perturbations resulting in a milder phenotype similar to EBS-WC or EBS-K.

5.6 Assessing the Functional Significance of Keratin Gene Mutations

There are several ways to predict and assess how a particular mutation may effect keratin filament assembly and the overall functioning of the cyoskeleton. The effect of an amino acid change on the secondary structure can be predicted from analysis of the

normal and mutant amino acid sequences using algorithms or, by studying the location of the mutations in relation to interaction with other subunits in neighbouring molecules during filament assembly or, from information from deletion and point mutagenesis studies. Deletion and point mutagenesis studies have already established the role of several of the keratin domains, and the effect of various amino acid substitutions within the rod, on the integrity of the keratin filament network *in vivo* and filament assembly *in vitro*. Keratin constructs containing an identified mutation or synthetic peptides to the mutation are two potential ways to test the functional significance of a particular mutation. However, when analysing mutants that fail to form normal filaments it may be difficult to establish which earlier assembly steps were successfully completed. It should also be noted that conflicting results may sometimes occur between *in vivo* and *in vitro* experiments, possibly due to the differences in conditions; for example, conditions *in vitro* such as the ionic strength can favour formation of filaments but may differ from those *in vivo*.

Functional studies, using keratin constructs containing some of the identified EBS-DM mutations, have confirmed that these single amino acid changes can disrupt the keratin filament network in a similar way to that observed in keratinocytes of EBS-DM patients (Coulombe *et al.*, 1991a; Letai *et al.*, 1993). Keratin constructs were made containing the arginine to cysteine and, arginine to histidine mutation at codon 125 of keratin 14 and the glutamic acid to glycine mutation in keratin 5 at codon 475. When transfected into human epidermal cells abnormal clumping of tonofilaments and the collapse of the keratin network was observed. These constructs were also unable to form normal 10 nm filaments *in vitro*. However, less dramatic effects were seen with the EBS-K leucine to proline mutation at codon 384 in keratin 14 which was still able to form filaments, although slightly shorter than wild type, and when the mutant was transfected into epithelial cells the majority of the transfected cells had an apparently normal keratin network (Letai *et al.*, 1993). These results were not too surprising;

although there have been some reports of abnormal filaments in cultured keratinocytes from EBS-K patients (Kitajima *et al.*, 1989; Letai *et al.*, 1993), the effects are much milder than the disorganisation in EBS-DM.

These results emphasise the different effects of these mutations when viewed in isolated cells compared to in the three dimensional structure of the epidermis, and this distinction should be taken into account when evaluating such studies. Keratin filament abnormalities were only just detectable by these *in vitro* assays with the EBS-K leucine384-proline mutation although, *in vivo*, this mutation resulted in widespread blistering. It has been suggested that the cyto-architecture of the cell is important in determining the susceptibility to lysis. Keratinocytes in culture are flatter than the cuboidal or columnar cells in a host tissue, and have a greater surface area to volume ratio that might be more resilient to cytolysis (Coulombe *et al.*, 1991b). It was predicted that the effects of mutations causing the milder Weber-Cockayne EBS would be difficult to investigate by these types of *in vitro* experiments since, keratinocytes from these patients generally have a normal appearing keratin network with no tonofilament clumping. However, since completing this thesis Chan *et al.* (1994) report two new mutations in Weber-Cockayne in the L12 linker of keratin 5 and have shown by filament assembly studies the subtle but significant effects of these mutations.

Synthetic peptides to some of the keratin 1 and keratin 10 gene mutations causing BCIE (Chipev *et al.*, 1992;1994), have been examined quantitatively for their ability to disassemble keratin filaments. Peptides containing the disease causing mutations were less efficient in disassembling preformed keratin filaments *in vitro* than the wild type peptides. This is probably because, due to the mutation, their structure is altered and so they cannot compete as effectively with the native keratin sequence.

5.7 Keratin Filament Assembly and the Location of Keratin Gene Mutations

From the two dimensional model proposed by Steinert for the packing of keratin filaments during filament assembly, one can predict why mutations in certain regions of the keratin molecule may be particularly disruptive to the filament network. Keratin heterodimers are thought to be arranged in antiparallel rows of in register and staggered molecules. Staggered molecules aligned in either of the two proposed ways (figure 47) are predicted to overlap with the adjacent molecule lying in the same direction; the last ten residues of the 2B domain of one molecule overlap with the first ten or so residues of the 1A domain of the neighbouring molecule (Steinert, 1993). Examination of these models reveals that several sequences overlap with each other; the beginning of the 1A domain with the end of 2B; 1A with the middle of the rod, near the L2 linker; the 2B domain with the L2 linker; the H1 and the H2 (type II keratins only) overlap with the beginning of 1A, the end of 2B and the L2 linker (figure 47). These regions are all highly conserved throughout intermediate filaments indicating that they have an important function in the filament network. A further highly conserved region common to all intermediate filaments is the stutter region near the centre of the α -helical 2B domain (Conway & Parry, 1988).

The function of these conserved regions has been tested using synthetic peptides corresponding to the H1, the beginning of the 1A and the end of the 2B domain (Hatzfeld & Weber, 1992; Steinert *et al.*, 1993). These peptides caused disassembly of preformed filaments *in vitro* and, when microinjected into epithelial cells, led to disassembly of the keratin filament networks. The peptides compete with and displace the keratin chains causing collapse of the filament network; this effect was reversible on removal of the peptide. Peptides from other regions of the keratin molecule and from unrelated proteins did not promote disassembly.

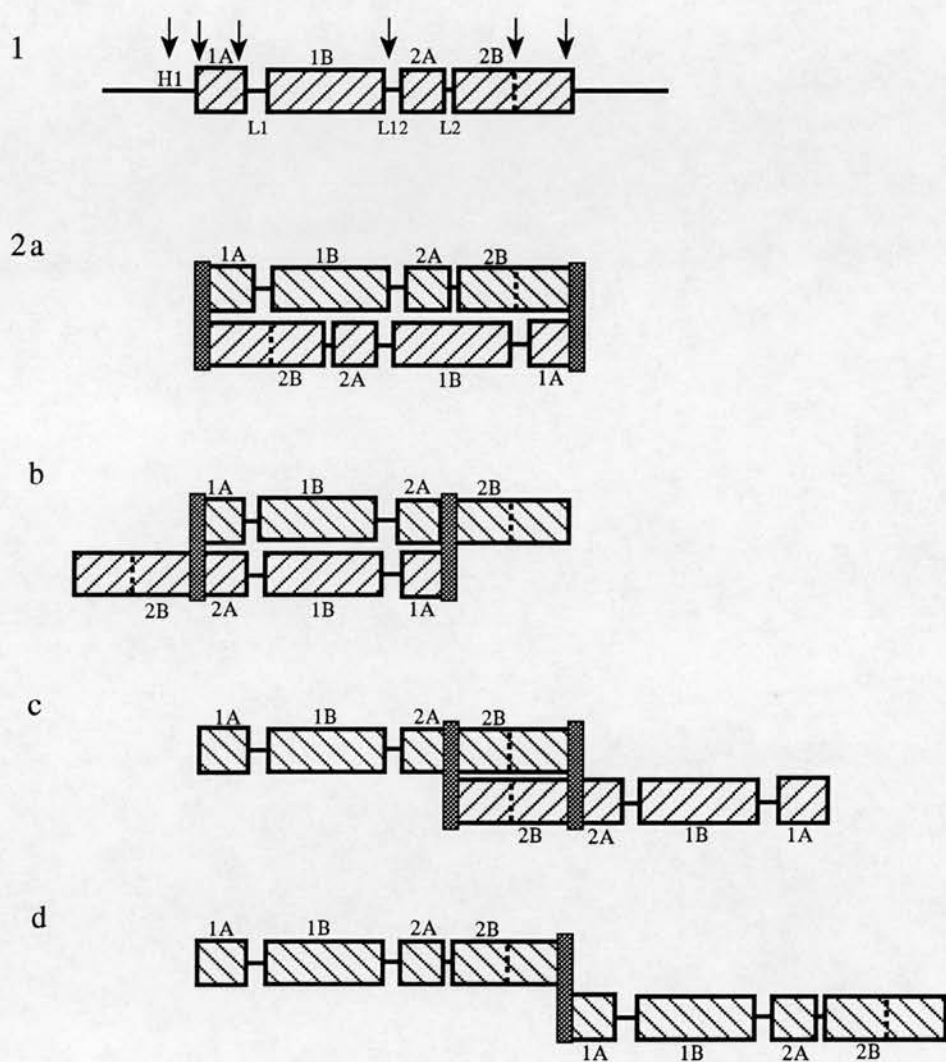


Figure 47. 1 Diagrammatic representation of a keratin dimer showing the position of the six mutation cluster sites, marked by arrows.

2a, b, c and d. The predicted modes of alignment of dimers during filament assembly. The shaded bars show the highly conserved sequence regions that frequently overlap with each other. (The non-helical terminal domains have been omitted).

A role for the non-helical H1 and H2 domains, in stabilising and determining the correct alignment of adjacent molecules was proposed (Steinert & Parry, 1993). The type I keratins with only a small H1 domain and no H2 domain depend on these domains of the type II keratin for correct alignment of neighbouring molecules, thereby explaining why both types of keratins are required for keratin filament assembly. Although a type II homodimer could form, a type I-type II heterodimer is predicted to be more thermodynamically stable (Steinert, 1990).

Interestingly, many of the mutations identified in either EBS or BCIE have been located in these highly conserved overlap regions supporting the proposal that these domains are important in maintaining the overall structure of the filament network. However, the mutations in the L12 linker region, in families A and B, identified a new mutational cluster site in an unexpected region. This demonstrates that mutations outside these overlap regions can also interfere with the filament network at some stage in the assembly process. Little is known about how the filaments fold in a three dimensional structure. Perhaps mutations in some of these other regions prevent the correct alignment or interaction of filaments in a three dimensional structure.

The clustering of pathological mutations at six sites (figure 47) would suggest that the sites where sequence variation is particularly disruptive to keratin filament assembly and stability are defined and limited. However, within these regions alteration of certain residues are more detrimental than others to the integrity of the cytoskeleton. The difference in disease severity caused by mutations within two residues of each other in the L12 linker of keratin 14 has already been mentioned. Mutations may also occur at noncritical sites between these cluster sites with no pathological consequences. It is known that polymorphisms occur within the keratin genes and some of these may explain the variation in skin resilience within the population. It is also possible that there may be sites where mutations are lethal. Until the total number of identified

mutations increases it is difficult to conclude any real significance from the clustering of mutations. However, with these six sites and certain “hot spot” residues already identified, these regions are likely to be targeted by the various groups studying EBS and BCIE in the screening of new patients and should identify any further cluster sites or particular mutation “hot spot” residues.

A point to consider is how many of these mutations involving the same nucleotide base change were caused by the same original mutation. Already, three Scottish EBS-WC families, thought originally to be unrelated, were found to be distantly related after identification of the same mutation in all three, combined with detailed family histories (Rugg *et al.*, 1993a).

5.8 Are Keratin Gene Mutations Responsible for all Cases of EBS and BCIE ?

The extent to which all variants of EBS are genetically related is unknown, although at least one subtype, EBS-Ogna, is probably not caused by a keratin defect. The EBS Ogna subtype has been linked to the erythrocyte enzyme, glutamic pyruvic transaminase at locus EBS1 at the long arm of chromosome 8 (Olaisen, 1973). It is also unknown whether mutations in the basal cell keratins will underlie all EBS-DM, EBS-K and EBS-WC cases and in time, further investigations, will probably determine the causes of some of the other rare subtypes of EBS. There are some EBS-DM and EBS-WC cases where keratin gene mutations have not yet been identified, such as family E. It remains to be seen, therefore, whether there are still unidentified mutation cluster sites, whether mutations will be found throughout the rod domain, or whether defects in genes other than those coding for keratin 5 or keratin 14 are responsible for these cases. It is quite possible that mutations in other genes, that are important in a structural or regulatory role in keratin filament assembly, could lead to the same clinical phenotype. Likely candidate genes include minor keratins such as keratin 9, which is

expressed in the palms and soles or a keratin-associated protein such as desmoplakin, which is known to be able to disrupt intermediate filament networks (Stappenbeck & Green, 1992). There could even be a combination of abnormalities. All the keratin gene mutations identified so far involve alterations, either nucleotide substitutions or deletions, to the primary amino acid sequence. It is also possible that alterations may occur at a higher level of the filament assembly process, although these might be expected to produce more subtle effects than those affecting the early stages of filament assembly.

In a similar way, whether all cases of BCIE are due to mutations in either of the suprabasal keratins K1 or K10, is as yet unknown. Other possible candidate genes include other keratins expressed in the suprabasal cells of stratifying epithelia, K2, K9 and K11, the envelope protein filaggrin (mapped to chromosome 1q; McKinley-Grant *et al.*, 1989) which is involved in terminal differentiation, the gene for epidermal transglutaminase on chromosome 14 which catalyses intermolecular cross linking of keratins necessary for proper stratification of skin (Polakowska *et al.*, 1991), or the cornified envelope precursor involucrin.

5.9 Other Diseases Involving Intermediate Filament Abnormalities

The identification of these pathological keratin gene mutations raises the question as to whether defects in intermediate filaments or intermediate filament-associated proteins are responsible for other diseases. Keratin gene mutations have now been identified in two other skin disorders, epidermolytic palmo-plantar hyperkeratosis (EPPK) and Ichthyosis bullosa of Siemens (IBS). EPPK is characterised by hyperkeratosis of palms and soles making keratin 9, which is expressed only in differentiating epidermis of palms and soles an obvious target gene. Recent studies have mapped the disease to type I keratins on chromosome 17 (Reis *et al.*, 1992, Matsumura *et al.*, 1993) and point

mutations have now been identified in the keratin 9 gene, in several families. Four different mutations have been found, all in the highly conserved helix initiation peptide (Reis *et al.*, 1994; Torchard *et al.*, 1994). Two of these mutations are at codon 162, resulting in an arginine to glutamine and an arginine to tryptophan amino acid change. The latter mutation was identified in five unrelated families (Reis *et al.*, 1994). Interestingly, this residue corresponds to the arginine in keratin 14 and keratin 10 that is also altered in some cases of EBS-DM and BCIE, indicating that this residue is important for the overall functioning of at least these three keratins and possibly others. IBS, characterised by symptoms similar but milder than those of BCIE has recently been linked to mutations in the keratin K2e gene (McLean *et al.*, 1994).

Intermediate filaments are present in all cells of the body but whether they all have a structural function is unknown. Diseases other than those affecting the skin, may also be caused by intermediate filament abnormalities. One such disorder is amyotrophic lateral sclerosis, a motor neuron disease, involving the type IV intermediate filaments, neurofilaments. It has recently been shown that over expression of neurofilament subunits in transgenic mice result in a phenotype resembling this disease (Cote *et al.*, 1993; Xu *et al.*, 1993). Excessive accumulation of neurofilaments and the progressive development of neurological defects were observed. Another disease that may involve alterations to intermediate filaments is Alzheimer's disease. In a similar way to EBS and BCIE, disorganisation and tangles of intermediate filaments are observed in neural cells, but involving neurofilaments rather than keratin filaments. Although Alzheimer's disease may be caused by a defect in β amyloid (Wasco *et al.*, 1993), the formation of neurofibrillary tangles may contribute to the overall cell degeneration.

Recent Findings in Dystrophic and Junctional EB

The pathogenesis of the other two main types of EB, dystrophic and junctional are both very different from the simplex form of EB and neither involve intermediate filament abnormalities. Investigations by several research groups to identify the cause of these two types has also progressed rapidly during the course of my project making it an exciting time for those affected by, or involved with, any of the three main types of EB. Both types of dystrophic EB, recessive and dominant, are associated with anchoring fibril abnormalities. The COL7A1 gene for type VII collagen, which is the major component of anchoring fibrils, has been linked to both the dominant form (Ryynanen *et al.*, 1991a; Al-Imara *et al.*, 1992; Gruis *et al.*, 1992; Ryynanen *et al.*, 1992) and the recessive form of DEB (Hovnanian *et al.*, 1992). Further studies have now identified mutations in the COL7A1 gene in some families affected by recessive DEB (Christiano *et al.*, 1993; Hilal *et al.*, 1993a,b).

The genetic defect responsible for some forms of junctional EB has also recently been identified. Abnormal expression in some JEB individuals, of certain intra-lamina lucida antigens of the epidermal basement membrane, AA3 (Kennedy *et al.*, 1985), GB3 (Heagerty *et al.*, 1986; Schofield *et al.*, 1990; Verrando *et al.*, 1991) and 19-DEJ (Fine, 1990), suggested that epitopes recognised by these antibodies may be directly involved in the pathogenesis of JEB. A recent study showed that the protein nicein/kalinin/BM600, present in the lamina lucida and recognised by GB3, is synthesised in the severe Herlitz variant of JEB, but with some structural alteration (Verrando *et al.*, 1993; Baudoin *et al.*, 1994). Pulkkinen *et al.* (1994) have since reported mutations in the gamma 2 chain gene of kalinin/laminin 5 as being responsible for some forms of JEB.

5.10 Prospects for EB Simplex Patients

The significance to those suffering from EBS of the identification of keratin gene mutations in some families is not only that the cause is now known, which in itself, with recent publications, has raised the profile of the disease, but there is now a realistic hope that some form of treatment could eventually become available. It is also likely that more specific prenatal diagnostic tests will be developed that are less invasive and can be performed earlier than at present.

Although EBS is generally considered mild enough not to warrant prenatal diagnosis, for some cases at risk from the most severe form, Dowling-Meara, it may be justified. At present, foetal skin biopsies, obtained at 16-18 weeks gestation, are examined for the ultrastructural level of skin cleavage and abnormal morphology such as keratin filament clumping in EBS-DM (Holbrook *et al.*, 1993). However, with the identification of keratin gene mutations in EBS and BCIE, it may be possible, on a molecular basis to diagnose these disorders at a very early stage of development as the keratins, K5, K14, K1 and K10 are present as early as six weeks gestation. Genetic linkage to specific genes or identification of a mutation in an affected parent will allow specific probes to be used for diagnosis in a particular family. DNA can be extracted from chorionic villi, which are easily obtainable at 10-12 weeks gestation and therefore, will allow diagnosis to be made earlier than is currently possible by a skin biopsy. One case of prenatal diagnosis by identification of a specific keratin mutation has now been reported for BCIE (Rothnagel *et al.*, 1994). Following the identification of a mutation in the DNA of the affected father, DNA extracted from chorionic villi samples, at 15 weeks gestation, from twin foetuses were examined by DNA sequencing. Both were identified as being heterozygous for the mutant gene, and this was confirmed by ultrastructural analysis of epidermal biopsies at 18 weeks gestation. It would also be possible, by DNA sequencing, to screen *in vitro* fertilised embryos from affected couples prior to implantation for an identified mutation.

5.11 Gene Therapy Prospects for EB Simplex

There are, as yet, no effective therapies to treat the clinical symptoms of EBS. A possible alternative to conventional drugs, for the treatment of a disease, is gene therapy. Although still in the early stages of development, clinical trials for certain disorders are now under way, and rapid progress is expected in this field in the next few years. Gene therapy involves the deliberate alteration of the genetic material of living cells to prevent or treat disease; the genetic defect is corrected rather than the symptoms of the disease being treated (Kessler *et al.*, 1993). Alteration or insertion of a new gene into target cells enables those cells to produce the required protein and thereby alleviate the disease (Vogel, 1993). Hereditary diseases, such as EBS, that have been identified as caused by defects in single genes, and are not treatable with conventional drugs, could potentially be treated by gene therapy. The first human gene therapy trial began in 1990 with the transfer of an adenosine deaminase (ADA) gene into lymphocytes of a patient with a severe immune deficiency, due to a lethal defect in this enzyme (Miller, 1992). More recently the first human trials using liposomes, instead of viruses for gene transfer, have begun for the treatment of cystic fibrosis. The gene, which when defective causes cystic fibrosis, is wrapped in liposomes, which fuse with the cell membrane and discharge the gene into the cell (Dickson, 1993).

For ethical and practical reasons somatic cell therapy rather than germline therapy is considered (Miller, 1990). A critical step in gene therapy is the method of delivering the genes into the target cells. Efficient delivery of the new gene, together with persistent expression of the gene in cells that proliferate for the lifetime of the patient, are required to provide a continuous and lifelong supply of the replaced gene product (St Louis & Verma, 1988; Miller, 1990). Most of the human gene trials use a combination of somatic cell therapy and gene therapy, where the target cells are removed from the patient and undergo genetic manipulation *ex vivo* and then the modified cells are transferred back to the patient (Mulligan, 1993). Modified or

attenuated viruses such as retroviruses or adeno-associated viruses are used as vectors to transfer the desired DNA sequences into the chromosomal DNA of the target cell. Alternatively the gene can be directly introduced into the target cell without removing the cells from the patient for manipulation, but this is less efficient than the *ex vivo* method as a lower percentage of cells contain the inserted gene (Vogel, 1993). Other delivery methods require introduction of the DNA sequences into the nucleus in an unintegrated form and include DNA liposome mixtures, directly administered DNA, and DNA combined with a targeted delivery system (for example, a monoclonal antibody or cellular-receptor targeted ligand-DNA conjugate) (Kessler *et al.*, 1993). Another potential way of delivering the gene is by particle bombardment, where an electric discharge device accelerates DNA-coated microscopic gold particles into the target tissues (Cheng *et al.*, 1993). An alternative to somatic gene therapy has been suggested by Tai and Sun (1993) that involves immuno-isolating genetically modified cells in a biocompatible membrane. This would provide a sustained delivery of the desired gene product and would allow nonsyngenic recipients to receive the same engineered cell line.

Keratinocytes are a potential target cell for gene therapy treatment. They are readily accessible, can be cultured to provide large sheets of cells, and grafting procedures are well established and have been successful in treatment of burns patients. Cultured keratinocytes could be transfected with the new gene *in vitro* and the modified keratinocytes grafted back onto the patients. The graft could be easily monitored and removed if necessary. As well as the potential use in the treatment of skin diseases with a keratinocyte defect, they could be used as target cells for the treatment of some systemic diseases, providing the secreted enzyme or factor of the inserted gene could pass through the epidermal basement membrane into the systemic system (Vogel, 1993). A point to consider is whether when a protein of a transgene is expressed at

high levels in differentiating keratinocytes, there will be any affect to the normal differentiation process.

With the recent identification of the genetic defects in several epidermal diseases it is now possible that gene therapy could, in the future, be used to alleviate some of the symptoms. In diseases that are inherited in an autosomal recessive manner, insertion of a normal copy of the affected gene would be likely to restore normal gene function. For example, in recessive DEB, where some cases are now known to be due to homozygous mutations in the type VII collagen gene, insertion of a normal copy of this gene would be expected to result in production of normal collagen VII.

However autosomal dominant disorders such as EBS present an additional problem. Although a normal copy of the gene is expressed in the affected tissues the mutant gene product exhibits dominant negative effects. Therefore, even if a normal keratin gene was inserted, the mutant protein would still be produced and interfere with normal keratin filament formation. In order to restore normal function, selective and specific deletion of the mutant gene is required. Although feasible, removal of a gene is more difficult than insertion of a gene. The two genes, mutant and normal, must be distinguishable by different target specificities. If a mutant keratin gene could be deleted it is expected that the remaining normal gene would produce enough normal keratin to provide a fully functioning keratin filament network, thereby correcting the defect.

There are several ways in which it may be possible to correct a keratin defect in EBS once the mutation specific to that family has been identified. These possibilities need to be thoroughly investigated *in vitro*, in cultured keratinocytes containing identified mutations, to establish the possiblity of developing such a technique for clinical use. Two ways by which a keratin defect may be corrected are by selective knockout of the

mutant keratin gene with antisense oligonucleotides or, the replacement of both the normal and mutant keratin genes with an “artificial” keratin gene, based on a retroviral system. Oligodeoxyribonucleotides can selectively inhibit gene expression and are short stranded DNA molecules that hybridize to complementary sequences in a target gene mRNA or associated sequence to interfere with and prevent formation of the gene product (Noonberg *et al.*, 1993). It may be possible to selectively inactivate a mutant keratin mRNA using an allele-specific synthetic antisense oligonucleotide specific for the keratin mutation. Application of such oligonucleotides to cultured keratinocytes, from affected individuals that have been immortalised and still carry the mutation, might selectively down-regulate the mutant keratin gene. However, the success of any down regulation would be difficult to detect by SDS polyacrylamide gels and immunoblotting as the normal and mutant keratins usually have identical electrophoretic mobilities. However, some families have been shown to have the K5a/5b polymorphism allowing the two K5 alleles to be separated due to the different electrophoretic migration rates of the K5a and K5b alleles. In families with a keratin 5 mutation where affected members are also heterozygous for the K5a/5b polymorphism it would therefore be possible to distinguish between the normal and mutant keratin. This would be an ideal system for initial studies to investigate “knock out” of the mutant keratin. If the mutant keratin was down regulated, keratinocyte cultures would need to be studied by immunofluorescence and electron microscopy to ensure that normal keratin filament networks had been restored.

If these types of experiments were successful in cell culture the ultimate goal would be to develop these strategies for clinical use. Although it will probably not be practical to treat extensive areas of the epidermis, many of these diseases affect only limited areas of the body which could potentially be treated. In principle, keratinocytes could be cultured from the patient, modified *in vitro* and grafted back onto the patient, or alternatively it may become possible to directly apply therapeutic oligonucleotides either

topically or by another route. Rapid progress is expected in the next few years in the application of gene therapy techniques to treat various diseases.

5.12 Conclusion and Future Work

This project, together with studies by other researchers in Dundee (Cell Structure Research Group, University of Dundee) and at centres throughout the world, has resulted in the recent and rapid progress in understanding the molecular basis of EBS. The genetic defect has now been identified in a number of pedigrees. Of the many inherited disorders, this now means EBS is one of the relatively few for which the genetic defect is known. In addition, these investigations have increased our knowledge about the structure and functioning of keratin intermediate filaments.

However, there are still many unanswered questions. Why are there so few extracutaneous manifestations in EBS when keratin 5 and keratin 14 are present in all stratified epithelia? Why is blistering more severe in Weber-Cockayne and Koebner variants in warmer weather, but not in the Dowling-Meara variant of EBS? Why do the blisters in Dowling-Meara occur in groups? Why the apparent improvement with age? Why is there sometimes a wide variation in severity within members of the same family? Variation in severity can also occur within BCIE families, and in one BCIE family, insertional polymorphisms in the V2 subdomains of the non-helical tails of keratins K1 and K10 were investigated as a possible explanation for the phenotypic variation (McLean *et al.*, 1994). Although there was an insertional polymorphism in the V2 domain of keratin 10, variation in the severity of the disease did not segregate with this polymorphism, thereby excluding this as the reason for the phenotypic heterogeneity in this particular family. It is possible that these phenotypic variations could be due to the interaction with other, as yet unidentified, factors.

The identification of mutations in further families and investigations on the effects of keratin gene mutations on cultured keratinocytes may provide answers to some of these questions. I am going to continue my interest in this area with a future project (at Dundee University and funded by DEBRA), to develop a way of correcting identified mutations in keratinocytes cultured from EBS patients. To achieve this, the two approaches already described, either selective deactivation of the mutant keratin gene or replacement of both the normal and mutant endogenous keratin genes with an "artificial" keratin gene, will be investigated. These studies will provide essential groundwork necessary for the development of EBS gene therapy.

CHAPTER 6

REFERENCES

Abanmi, A., Joshi, R.K., Atukorala, D.N., Pedersen, N.B. and Al Khamis, O. Autosomal recessive epidermolysis bullosa simplex. A case report. *British Journal of Dermatology* 130:115-117, 1994.

Albers, K. and Fuchs, E. The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines. *Journal of Cell Biology* 105:791-806, 1987.

Albers, K. and Fuchs, E. Expression of mutant keratin cDNAs in epithelial cells reveals possible mechanisms for initiation and assembly of intermediate filaments. *Journal of Cell Biology* 108:1477-1493, 1989.

Alley, M.R., O'Hara, P.J. and Middelberg, A. An epidermolysis bullosa of sheep. *New Zealand Veterinary Journal* 22:55-59, 1974.

Al-Lmara, L., Richards, A.J., Eady, R.A., Leigh, I.M., Farrall, M. and Pope, F.M. Linkage of autosomal dominant dystrophic epidermolysis bullosa in three British families to the marker D3S2 close to the COL7A1. *Journal of Medical Genetics* 29:381-382, 1992.

Alper, J.C., Baden, H.P. and Goldsmith R.G. Kindler's syndrome. *Archives of Dermatology* 114:457, 1978.

Andreano, J.M. and Tomecki, K.J. Epidermolysis bullosa simplex responding to isotretinoin (letter). *Archives of Dermatology* 124:1445-1446, 1988.

Anton-Lamprecht, I. Prenatal diagnosis of genetic disorders of the skin by means of electron microscopy. *Human Genetics* 59:392-405, 1981.

Anton-Lamprecht, I. and Schnyder, U.W. Epidermolysis bullosa herpetiformis Dowling-Meara. Report of a case and pathomorphogenesis. *Dermatologica* 164:221-235, 1982.

Anton-Lamprecht, I., Gedde-Dahl, T., Jr. and Schnyder, U.W. Ultrastructural characterization of a new dominant epidermolysis genotype (abstract). *Journal of Investigative Dermatology* 72:280 1979.

Anton-Lamprecht, I., Rauskolb, R., Jovanovic, V., Kern, B., Arnold, M.L. and Schenck, W. Prenatal diagnosis of epidermolysis bullosa dystrophica Hallopeau-Siemens with electron microscopy of fetal skin. *Lancet* 2:1077-1079, 1981.

Bader, B.L., Magin, T.M., Freudenmann, M., Stumpp, S. and Franke, W.W. Intermediate filaments formed de novo from tail-less cytokeratins in the cytoplasm and in the nucleus. *Journal of Cell Biology* 115:1293-1307, 1991.

Bader, B.L., Magin, T.M., Hatzfeld, M. and Franke, W.W. Amino acid sequence and gene organization of cytokeratin no. 19, an exceptional tail-less intermediate filament protein. *Embo Journal* 5:1865-1875, 1986.

Bajkowski, A.S. and Frankfater, A. Specific spectrophotometric assays for cathepsin B1. *Analytical Biochemistry* 68:119-127, 1975.

Baribault, H. and Oshima, R.G. Polarized and functional epithelia can form after the targeted inactivation of both mouse keratin 8 alleles. *Journal of Cell Biology* 115:1675-1684, 1991.

- Bart, B.J. Epidermolysis bullosa and congenital localized absence of skin. *Archives of Dermatology* 101:78-81, 1970.
- Bart, B.J., Gorlin, R.J., Anderson, V.E. and Lynch, F.W. Congenital localized absence of skin and associated abnormalities resembling epidermolysis bullosa. A new syndrome. *Archives of Dermatology* 93:296-304, 1966.
- Bassett, H. A congenital bovine epidermolysis resembling epidermolysis bullosa simplex of man. *Veterinary Record* 121:8-11, 1987.
- Baudoin, C., Miquel, C., Blanchet-Bardon, C., Gambini, C., Meneguzzi, G. and Ortonne, J. Herlitz junctional epidermolysis bullosa keratinocytes display heterogeneous defects of nicein/kalinin gene expression. *Journal of Clinical Investigations* 93:862-869, 1994.
- Bauer, E.A. and Briggaman, R.A. Hereditary epidermolysis bullosa. *Dermatology in General Medicine I, 4th Ed*, eds. Fitzpatrick, T.B., Eisen, A.Z., Wolff, K., Freedberg, I.M. and Austen, K.F. McGraw Hill, Inc. 654-669, 1993.
- Bergstresser, P.R. and Taylor, J.R. Epidermal 'turnover time'--a new examination. *British Journal of Dermatology* 96:503-509, 1977.
- Berns, M.W. The diversity of cells. *Cells 2nd Ed*. Sanders College Publishing. 4, 1983.
- Beynon, R.J. and Bond, J.S. *Proteolytic Enzymes: A Practical Approach*. IRL Oxford University Press. 1989.
- Blanchet-Bardon, C., Dumez, Y., Nazzaro, V., Mimoz, C. and Puissant, A. Antenatal diagnosis of hereditary epidermolysis bullosa. *Annales De Dermatologie Et De Venereologie* 114:525-539, 1987.
- Blanchet-Bardon, C., Souteyrand, P., Mimoz, C., Malpuech, G. and Dumez, Y. Epidermolysis bullosa letalis. Antenatal exclusion diagnosis (letter). *Presse Medicale - Paris* 13:680-681, 1984.
- Bonifas, J.M., Rothman, A.L. and Epstein, E.H., Jr. Linkage of epidermolysis bullosa simplex to probes in the region of keratin gene clusters on chromosomes 12q and 17q (abstract). *Journal of Investigative Dermatology* 96:550, 1991a.
- Bonifas, J.M., Rothman, A.L. and Epstein, E.H., Jr. Epidermolysis bullosa simplex: evidence in two families for keratin gene abnormalities. *Science* 254:1202-1205, 1991b.
- Bonnetblanc, J.M. and Bouquier, J.J. Response to pipamperone in case of epidermolysis bullosa herpetiformis (letter). *Lancet* 1:1327-1328, 1986.
- Bordas, X., Palou, J., Capdevila, J.M. and Mascaro, J.M. Kindler's syndrome. Report of a case. *Journal of the American Academy of Dermatology* 6:263-265, 1982.
- Boss, J.M., Matthews, C.N., Peachey, R.D. and Summerly, R. Speckled hyperpigmentation, palmo-plantar punctate keratoses and childhood blistering: a clinical triad, with variable associations. A report of two families. *British Journal of Dermatology* 105:579-585, 1981.

Boyce, S.T. and Ham, R.G. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *Journal of Investigative Dermatology* 81:33s-40s, 1983.

Briggaman, R.A. Biochemical composition of the epidermal-dermal junction and other basement membrane. *Journal of Investigative Dermatology* 78:1-6, 1982.

Brow, M.A.D. *PCR Protocols: A Guide to Methods and Applications*, eds. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. Academic Press, New York. 194, 1990.

Bruckner-Tuderman, L., Guscetti, F. and Ehrensperger, F. Animal model for dermolytic mechanobullous disease: sheep with recessive dystrophic epidermolysis bullosa lack collagen VII. *Journal of Investigative Dermatology* 96:452-458, 1991.

Bruckner-Tuderman, L., Vogel, A., Ruegger, S., Odermatt, B., Tonz, O. and Schnyder, U.W. Epidermolysis bullosa simplex with mottled pigmentation. *Journal of the American Academy of Dermatology* 21:425-432, 1989.

Buchbinder, L.H., Lucky, A.W., Ballard, E., Stanley, J.R., Stolar, E., Tabas, M., Bauer, E.A. and Paller, A.S. Severe infantile epidermolysis bullosa simplex. Dowling-Meara type. *Archives of Dermatology* 122:190-198, 1986.

Butler, D.F., Berger, T.G., James, W.D., Smith, T.L., Stanley, J.R. and Rodman, O.G. Bart's syndrome: microscopic, ultrastructural, and immunofluorescent mapping features. *Pediatric Dermatology* 3:113-118, 1986.

Carlin, B., Jaffe, R. and Binder, B. Entactin, a novel basal lamina-associated sulphated glycoprotein. *Journal of Biological Chemistry* 256:5209-5214, 1981.

Carter, D.M. and Caldwell-Brown, D. The national epidermolysis bullosa registry. *Epidermolysis bullosa: A Comprehensive Review of Classification Management and Laboratory Studies*, eds. Priestley, G.C., Tidman, M.J., Weiss, J.B. and Eady, R.A. Crowthorne, England: Dystrophic Epidermolysis Bullosa Research Association. 173-175, 1990.

Carter, W.G., Ryan, M.C. and Gahr, P.J. Epiligrin, a new cell adhesion ligand for integrin $\alpha 3 \beta 1$ in epithelial basement membranes. *Cell* 65:599-610, 1991.

Chan, Y.M., Yu, Q.C., Fine, J.D. and Fuchs, E. The genetic basis of Weber-Cockayne epidermolysis bullosa simplex. *Proceedings of the National Academy of Sciences of the United States of America* 90:7414-7418, 1993.

Chan, Y.M., Yu, Q.C., LeBlanc-Straceski, J., Christiano, A., Pulkkinen, L., Kucherlapati, R.S., Uitto, J. and Fuchs, E. Mutations in the non-helical linker segment L1-2 of keratin 5 in patients with Weber-Cockayne epidermolysis bullosa simplex. *Journal of Cell Science* 107:765-774, 1994.

Chen, M.A., Bonifas, J.M., Matsumura, K., Blumenfeld, A. and Epstein, E.H., Jr. A novel three-nucleotide deletion in the helix 2B region of keratin 14 in epidermolysis bullosa simplex: $\Delta E375$. *Human Molecular Genetics* 2:1971-1972, 1993.

Cheng, J., Syder, A.J., Yu, Q.C., Letai, A., Paller, A.S. and Fuchs, E. The genetic basis of epidermolytic hyperkeratosis: a disorder of differentiation-specific epidermal keratin genes. *Cell* 70:811-819, 1992.

- Cheng, L., Ziegelhoffer, P.R. and Yang, N. *In vitro* promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. *Proceedings of the National Academy of Sciences of the United States of America* 90:4455-4459, 1993.
- Chipev, C.C., Korge, B.P., Markova, N., Bale, S.J., DiGiovanna, J.J., Compton, J.G. and Steinert, P.M. A leucine---proline mutation in the H1 subdomain of keratin 1 causes epidermolytic hyperkeratosis. *Cell* 70:821-828, 1992.
- Chipev, C.C., Yang, J-M, DiGiovanna, J.J., Steinert, P.M., Marekov, L., Compton, J.G. and Bale, S.J. Preferential sites in keratin 10 that are mutated in epidermolytic hyperkeratosis. *American Journal of Human Genetics* 54:179-190, 1994.
- Chou, P.Y. and Fasman, G.D. Prediction of the secondary structure of proteins from their amino acid sequence. *Advances In Enzymology & Related Areas of Molecular Biology* 47:45-148, 1978.
- Chou, Y.H., Bischof, J.R., Beach, D. and Goldman, R.D. Intermediate filament reorganization during mitosis is mediated by p34^{cdc2} phosphorylation of vimentin. *Cell* 62:1063-1071, 1990.
- Christiano, A.M., Greenspan, D.S., Hoffman, G.G., Zhang, X., Tamai, Y., Lin, A.N., Dietz, H.C., Hovnanian, A. and Uitto, J. A missense mutation in type VII collagen in two affected siblings with recessive dystrophic epidermolysis bullosa. *Nature Genetics* 4:62-66, 1993.
- Cockayne, E.A. Recurrent bullous eruption of feet. *British Journal of Dermatology* 50:358-362, 1938.
- Coleman, R., Harper, J.I. and Lake, B.D. Epidermolysis bullosa simplex with mottled pigmentation. *British Journal of Dermatology* 128:679-685, 1993.
- Collin, C., Moll, R., Kubicka, S., Ouhayoun, J.P. and Franke, W.W. Characterization of human cytokeratin 2, an epidermal cytoskeletal protein synthesized late during differentiation. *Experimental Cell Research* 202:132-141, 1992a.
- Collin, C., Ouhayoun, J.P., Grund, C. and Franke, W.W. Suprabasal marker proteins distinguishing keratinizing squamous epithelia: cytokeratin 2 polypeptides of oral masticatory epithelium and epidermis are different. *Differentiation* 51:137-148, 1992b.
- Conway, J.F. and Parry, D.A. Intermediate filament structure: 3 Analysis of sequence homologies. *International Journal of Biological Macromolecules* 10:79-98, 1988.
- Cooper, D., Schermer, A. and Sun, T.T. Classification of human epithelia and their neoplasms using monoclonal antibodies to keratins: strategies, applications, and limitations. *Laboratory Investigation* 52:243-256, 1985.
- Cooper, D.N. and Youssoufian, H. The CpG dinucleotide and human genetic disease. *Human Genetics* 78:151-155, 1988.
- Cote, F., Collard, J-F. and Julien, J-P. Progressive neuronopathy in transgenic mice expressing the human neurofilament heavy gene: a mouse model of amyotrophic lateral sclerosis. *Cell* 73:35-46, 1993.
- Coulombe, P.A. The cellular and molecular biology of keratins: beginning a new era. *Current Opinion in Cell Biology* 5:17-29, 1993.

- Coulombe, P.A. and Fuchs, E. Elucidating the early stages of keratin filament assembly. *Journal of Cell Biology* 111:153-169, 1990.
- Coulombe, P.A., Chan, Y.M., Albers, K. and Fuchs, E. Deletions in epidermal keratins leading to alterations in filament organization *in vivo* and in intermediate filament assembly *in vitro*. *Journal of Cell Biology* 111:3049-3064, 1990.
- Coulombe, P.A., Hutton, M.E., Letai, A., Hebert, A., Paller, A.S. and Fuchs, E. Point mutations in human keratin 14 genes of epidermolysis bullosa simplex patients: genetic and functional analyses. *Cell* 66:1301-1311, 1991a.
- Coulombe, P.A., Hutton, M.E., Vassar, R. and Fuchs, E. A function for keratins and a common thread among different types of epidermolysis bullosa simplex diseases. *Journal of Cell Biology* 115:1661-1674, 1991b.
- Crewther, W.G., Inglis, A.S. and McKern, N.M. Amino acid sequence of α -helical segments from S-carboxymethylkerateine A. *Biochemical Journal* 173:365-371, 1978.
- Crick, F.H.C. The packing of α -helices: simple coiled coils. *Acta Cryst.* 6:689-697, 1953.
- Davison, B.C.C. Epidermolysis bullosa. *Journal of Medical Genetics* 2:233-242, 1965.
- DesGroseilliers, J.P. and Brisson, P. Localized epidermolysis bullosa. Report of two cases and evaluation of therapy with glutaraldehyde. *Archives of Dermatology* 109:70-72, 1974.
- Dickson, D. UK scientists test liposome gene therapy technique. *Nature* 365:4, 1993.
- Dong, W., Ryynanen, M. and Uitto, J. Identification of a leucine-to-proline mutation in the keratin 5 gene in a family with the generalized Kobner type of epidermolysis bullosa simplex. *Human Mutation* 2:94-102, 1993.
- Dowling, G.B. and Meara, R.H. Epidermolysis bullosa resembling juvenile dermatitis herpetiformis. *British Journal of Dermatology* 66:139-143, 1954.
- Dunstan, R.W., Sills, R.C., Wilkinson, J.E., Paller, A.S. and Hashimoto, K.H. A disease resembling junctional epidermolysis bullosa in a toy poodle. *American Journal of Dermatopathology* 10:442-447, 1988.
- Eby, J. Investigating presumed child abuse (letter). *Canadian Medical Association Journal* 139:8, 1988.
- Eckelt, A., Herrmann, H. and Franke, W.W. Assembly of a tail-less mutant of the intermediate filament protein, vimentin, *in vitro* and *in vivo*. *European Journal of Cell Biology* 58:319-330, 1992.
- Eckert, R.L. and Rorke, E.A. The sequence of the human epidermal 58-kD (:5) type II keratin reveals an absence of 5' upstream sequence conservation between coexpressed epidermal keratins. *DNA* 7:337-345, 1988.

- Eichner, R., Sun, T.T. and Aebi, U. The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments. *Journal of Cell Biology* 102:1767-1777, 1986.
- Eisenberg, M., Shorey, C.D. and de Chair-Baker, W. Epidermolysis bullosa--a new subgroup. *Australasian Journal of Dermatology* 27:15-18, 1986.
- Ellison, J. and Garrod, D.R. Anchoring filaments of the amphibian epidermal-dermal junction traverse the basal lamina entirely from the plasma membrane of hemidesmosomes to the dermis. *Journal of Cell Science* 72:163-172, 1984.
- Eriksson, J.E., Opal, P. and Goldman, R.D. Intermediate filament dynamics. *Current Opinion in Cell Biology* 4:99-104, 1992.
- Escurat, M., Phamgia, H., Huc, C., Pouplardbarthelaix, A., Boitard, C., Bach, J.F., Gros, F. and Portier, M.M. A new monoclonal antibody recognizing the amino-terminal consensus sequence of vertebrate intermediate filaments. *FEBS Letters* 253:157-162, 1989.
- Ferrari, S., Cannizzaro, L.A., Battini, R. Huebner, K. and Baserga, R. The gene encoding human vimentin is located on the short arm of chromosome 10. *Cytogenetics & Cell Genetics* 46: 614, 1987.
- Fine, J.D. Epidermolysis bullosa. Clinical aspects, pathology, and recent advances in research. *International Journal of Dermatology* 25:143-157, 1986.
- Fine, J.D. Changing clinical and laboratory concepts in inherited epidermolysis bullosa. *Archives of Dermatology* 124:523-526, 1988.
- Fine, J.D. 19-DEJ-1, a monoclonal antibody to the hemidesmosome-anchoring filament complex, is the only reliable immunohistochemical probe for all major forms of junctional epidermolysis bullosa. *Archives of Dermatology* 126:1187-1190, 1990.
- Fine, J.D. and Griffith, R.D. A specific defect in glycosylation of epidermal cell membranes. Definition in skin from patients with epidermolysis bullosa simplex. *Archives of Dermatology* 121:1292-1296, 1985.
- Fine, J.D. and Johnson, L. Evaluation of the efficacy of topical bufexamac in epidermolysis bullosa simplex. A double-blind placebo-controlled crossover trial. *Archives of Dermatology* 124:1669-1672, 1988.
- Fine, J.D., Bauer, E.A., Briggaman, R.A., Carter, D.M., Eady, R.A., Esterly, N.B., Holbrook, K.A., Hurwitz, S., Johnson, L., Lin, A., Pearson, R. and Sybert, V.P. Revised clinical and laboratory criteria for subtypes of inherited epidermolysis bullosa. A consensus report by the Subcommittee on Diagnosis and Classification of the National Epidermolysis Bullosa Registry. *Journal of the American Academy of Dermatology* 24:119-135, 1991.
- Fine, J.D., Johnson, L. and Wright, T. Epidermolysis bullosa simplex superficialis. A new variant of epidermolysis bullosa characterized by subcorneal skin cleavage mimicking peeling skin syndrome. *Archives of Dermatology* 125:633-638, 1989a.
- Fine, J.D., Johnson, L., Wright, T. and Horiguchi, Y. Epidermolysis bullosa simplex: identification of a kindred with autosomal recessive transmission of the Weber-Cockayne variety. *Pediatric Dermatology* 6:1-5, 1989b.

- Fine, J.D., Stenn, J., Johnson, L., Wright, T., Bock, H.G. and Horiguchi, Y. Autosomal recessive epidermolysis bullosa simplex. Generalized phenotypic features suggestive of junctional or dystrophic epidermolysis bullosa, and association with neuromuscular diseases. *Archives of Dermatology* 125:931-938, 1989c.
- Fine, J.D., Stewart, B and Austin, R. Epidermolysis bullosa blister fluid and human skin organ culture: an unreliable *in vitro* model for the disease (abstract). *Journal of Investigative Dermatology* 87:139, 1986.
- Fischer, T. and Gedde-Dahl, T., Jr. Epidermolysis bullosa simplex and mottled pigmentation: a new dominant syndrome. I. Clinical and histological features. *Clinical Genetics* 15:228-238, 1979.
- Foisner, R. and Wiche, G. Intermediate filament-associated proteins. *Current Opinion in Cell Biology* 3:75-81, 1991.
- Foisner, R., Leichtfried, F.E., Herrmann, H., Small, J.V., Lawson, D. and Wiche, G. Cytoskeleton-associated plectin: in situ localization, *in vitro* reconstitution, and binding to immobilized intermediate filament proteins. *Journal of Cell Biology* 106:723-733, 1988.
- Fraki, J.E., Schechter, N.M. and Lazarus, G.S. Serine proteinases in human cutaneous mastocytosis. *Archives of Dermatological Research* 278:363-366, 1986.
- Fuchs, E. Keratins as biochemical markers of epithelial differentiation. *Trends in Genetics* 4:277-281, 1988.
- Fuchs, E. Epidermal differentiation. *Current Opinion in Cell Biology* 2:1028-1035, 1990.
- Fuchs, E. Threads between useful and useless. *Current Biolgy* 1:284-286, 1991.
- Fuchs, E. and Green, H. The expression of keratin genes in epidermis and cultured epidermal cells. *Cell* 15:887-897, 1978.
- Fuchs, E. and Green, H. Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell* 19:1033-1042, 1980.
- Fuchs, E., Coppock, S.M., Green, H. and Cleveland, D.W. Two distinct classes of keratin genes and their evolutionary significance. *Cell* 27:75-84, 1981.
- Fuchs, E., Esteves, R.A. and Coulombe, P.A. Transgenic mice expressing a mutant keratin 10 gene reveal the likely genetic basis for epidermolytic hyperkeratosis. *Proceedings of the National Academy of Sciences of the United States of America* 89:6906-6910, 1992.
- Gamborg Nielsen, P. and Sjolund, E. Epidermolysis bullosa simplex localisata associated with anodontia, hair and nail disorders: a new syndrome. *Acta Dermato-Venereologica* 65:526-530, 1985.
- Garnier, J., Osguthorpe, D.J. and Robson, B. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *Journal of Molecular Biology* 120:97-120, 1978.
- Gedde-Dahl, T., Jr. *Epidermolysis bullosa: A Clinical, Genetic and Epidemiological Study*. The John Hopkins University Press, Baltimore. 1971.

Gedde-Dahl, T., Jr. Classification of epidermolysis bullosa. *Padiatrische Dermatologie*, ed. Herzberg, J.J. Schattauer Verlag, Stuttgart, New York. 65-91, 1978.

Gedde-Dahl, T., Jr. Sixteen types of epidermolysis bullosa. On the clinical discrimination, therapy and prenatal diagnosis. *Acta Dermato-Venereologica - Supplementum* 95:74-87, 1981.

Gedde-Dahl, T., Jr. and Anton-Lamprecht, I. Epidermolysis bullosa. *Principles and Practice of Medical Genetics I*, eds. Emery, A.E.H., and Rimoin, D.L. Churchill Livingston, Inc. Edinburgh, 672-687, 1983.

Geisler, N., Schunemann, J. and Weber, K. Chemical cross-linking indicates a staggered and antiparallel protofilament of desmin intermediate filaments and characterizes one higher-level complex between protofilaments. *European Journal of Biochemistry* 206:841-852, 1992.

Georgatos, S.D. and Blobel, G. Two distinct attachment sites for vimentin along the plasma membrane and the nuclear envelope in avian erythrocytes: a basis for a vectorial assembly of intermediate filaments. *Journal of Cell Biology* 105:105-115, 1987.

Giese, G. and Traub, P. Induction of vimentin synthesis in mouse myeloma cells MPC-11 by 12-0-tetradecanoylphorbol-13-acetate. *European Journal of Cell Biology* 40:266-274, 1986.

Gill, S.R., Wong, P.C., Monteiro, M.J. and Cleveland, D.W. Assembly properties of dominant and recessive mutations in the small mouse neurofilament (NF-L) subunit. *Journal of Cell Biology* 111:2005-2019, 1990.

Granek, H. and Baden, H.P. Corneal involvement in epidermolysis bullosa simplex. *Archives of Ophthalmology* 98:469-472, 1980.

Green, K.A., Parry, D.A., Sreinert, P.M., Virata, M.L., Wagner, R.M., Angst, B.D. and Nilles, L.A. Structure of the human desmoplakins: implications for function in the desmosomal plaque. *Journal of Biological Chemistry* 265:2603-2612, 1990.

Gruis, N.A., Bavinck, J.N., Steijlen, P.M., van der Schroeff, J.G., van Haeringen, A., Happle, R., Mariman, E., van Beersum, S.E., Uitto, J., Vermeer, B.J. and Frants, R.R. Genetic linkage between the collagen VII (COL7A1) gene and the autosomal dominant form of dystrophic epidermolysis bullosa in two Dutch kindreds. *Journal of Investigative Dermatology* 99:528-530, 1992.

Guelstein, V.I., Tchypysheva, T.A., Ermilova, V.D., Litvinova, L.V. and Troyanosky, S.M. Monoclonal antibody mapping of keratins 8 and 17 and of vimentin in normal human mammary gland, benign tumors, dysplasias and breast cancer. *International Journal of Cancer* 42:147-153, 1988.

Haber, R.M., Hanna, W., Ramsay, C.A. and Boxall, L.B. Hereditary epidermolysis bullosa. *Journal of the American Academy of Dermatology* 13:252-278, 1985.

Hachisuka, H., Nomura, H., Sakamoto, F. and Sasai, Y. Cytofluorometric study of lectin binding to the keratinocytes of epidermolysis bullosa simplex. *Acta Histochemica* 89:85-89, 1990.

Halprin, K.M. Epidermal "turnover time" - a re-examination. *British Journal of Dermatology* 86:14-19, 1972.

- Haneke, E. and Anton-Lamprecht, I. Ultrastructure of blister formation in epidermolysis bullosa hereditaria: V. Epidermolysis bullosa simplex localisata type Weber-Cockayne. *Journal of Investigative Dermatology* 78:219-223, 1982.
- Hanukoglu, I. and Fuchs, E. The cDNA sequence of a human epidermal keratin: divergence of sequence but conservation of structure among intermediate filament proteins. *Cell* 31:243-252, 1982.
- Hanukoglu, I. and Fuchs, E. The cDNA sequence of a type II cytoskeletal keratin reveals constant and variable structural domains among keratin. *Cell* 33:915-924, 1983.
- Harbury, P.B., Zhang, T., Kim, P.S. and Alber, T. A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science* 262:1401-1407, 1993.
- Hatzfeld, M. and Weber, K. The coiled coil of *in vitro* assembled keratin filaments is a heterodimer of type I and II keratins: use of a site specific mutagenesis and recombinant protein expression. *Journal of Cell Biology* 110:1199-1210, 1990a.
- Hatzfeld, M. and Weber, K. Tailless keratins assemble into regular intermediate filaments *in vitro*. *Journal of Cell Science* 97:317-324, 1990b.
- Hatzfeld, M. and Weber, K. Modulation of keratin intermediate filament assembly by single amino acid exchanges in the consensus sequence at the C-terminal end of the rod domain. *Journal of Cell Science* 99:351-362, 1991.
- Hatzfeld, M. and Weber, K. A synthetic peptide representing the consensus sequence motif at the carboxy-terminal end of the rod domain inhibits intermediate filament assembly and disassembles preformed filaments. *Journal of Cell Biology* 116:157-166, 1992.
- Hauge, 1962. Cited in: Gedde-Dahl, T., Jr. *Epidermolysis bullosa: A Clinical, Genetic and Epidemiological Study*. The John Hopkins University Press, Baltimore. 1971.
- Heagerty, A.H., Kennedy, A.R., Eady, R.A., Hsi, B.L., Verrando, P., Yeh, C.J. and Ortonne, J.P. GB3 monoclonal antibody for diagnosis of junctional epidermolysis bullosa (letter) *Lancet* 1:860, 1986.
- Hedberg, K.K. and Chen, L.B. Absence of intermediate filaments in a human adrenal cortex carcinoma-derived cell line. *Experimental Cell Research* 163:509-517, 1986.
- Heid, H.W., Werner, E. and Franke, W.W. The complement of native α -keratin polypeptides of hair forming cells: a subset of eight polypeptides that differ from epithelial cytokeratins. *Differentiation* 32, 101-119, 1986.
- Hilal, L., Hovnanian, A., Duquensnoy, P., Blanchet-Bardon, C., Christiano, A.M., Uitto, J. and Gossens, M. Characterization of mutations within the COL7A1 gene in patients with recessive dystrophic epidermolysis bullosa (abstract). *Journal of Investigative Dermatology* 100:499, 1993a.
- Hilal, L., Rochat, A. Duquesnoy, P., Blanchet-Bardon, C., Wechsler, J., Martin, N., Christiano, A.M., Barrandon, Y., Uitto, J., Goossens, M. and Hovnanian, A. A homozygous insertion-deletion in the type VII collagen gene (COL7A1) in Hallopeau-Siemens dystrophic epidermolysis bullosa. *Nature Genetics* 5:287-293, 1993b.

- Hinter, H., Stingl, G., Schuler, P., Fritsch, P., Stanley, J., Katz, S. and Wolff, K. Immunofluorescence mapping of antigenic determinants within the dermal-epidermal junction in mechanobullous diseases. *Journal of Investigative Dermatology* 76:113-118, 1981.
- Hoffman, M. A layer by layer look at the skin blister disease. *Science* 254:1111-1112, 1991.
- Holbrook, K.A., Wapner, R. and Jackson, L. Diagnosis and prenatal diagnosis of epidermolysis bullosa herpetiformis (Dowling Meara) in a mother, two affected children and an affected fetus. *Prenatal Diagnosis* 12:725-739, 1992.
- Holbrook, K.A., Smith, L.T. and Elias, S. Prenatal diagnosis of genetic skin disease using fetal skin biopsy samples. *Archives of Dermatology* 129:1437-1454, 1993.
- Horn, H.M., Eady, R.A.J. and Tidman, M.J. The national epidermolysis bullosa register in Scotland (abstract). *in press*
- Hovnanian, A., Duquesnoy, P., Blanchet-Bardon, C., Knowlton, R.G., Amselem, S., Lathrop, M., Dubertret, L., Uitto, J. and Goossens, M. Genetic linkage of recessive dystrophic epidermolysis bullosa to the type VII collagen gene. *Journal of Clinical Investigation* 90:1032-1036, 1992.
- Hovnanian, A., Pollack, E., Hilal, L., Rochat, A., Prost, C., Barradon, Y. and Goossens, M. A missense mutation in the rod domain of keratin 14 associated with recessive epidermolysis bullosa simplex. *Nature Genetics* 3:327-332, 1993.
- Hoyheim, B., Gedde-Dahl, T., Jr., Berg, E. and Olaisen, B. Linkage studies in epidermolysis bullosa simplex (abstract). *Journal of Investigative Dermatology* 98:397, 1992.
- Humphries, M.M., Sheils, D., Lawler, M., Farrar, G.J., McWilliam, P., Kenna, P., Bradley, D.G., Sharp, E.M., Gaffney, E.F., Young, M., Uitto, J. and Humphries, P. Epidermolysis bullosa: evidence for linkage to genetic markers on chromosome 1 in a family with the autosomal dominant simplex form. *Genomics* 7:377-381, 1990.
- Humphries, M.M., Sheils, D.M., Farrar, G.J., Kumar-Singh, R., Kenna, P.F., Mansergh, F.C., Jordan, S.A., Young, M. and Humphries, P. A mutation (Met \rightarrow Arg) in the type I keratin (K14) gene responsible for autosomal dominant epidermolysis bullosa simplex. *Human Mutation* 2:37-42, 1993.
- Hunter, J.A.A., McVittie, E. and Comaish, J.S. Light and electron microscopic studies of physical injury to the skin II. Friction. *British Journal of Dermatology* 90:491-499, 1974.
- Hurst, J., Flavell, D., Julien, J.P., Meijer, D., Mushynski, W. and Grosveld, F. The human neurofilament gene (NF-L) is located on the short arm of chromosome 8. *Cytogenetics & Cell Genetics* 45:30-32, 1987.
- Ikeda, S., Manabe, M., Muramatsu, T., Takamori, K. and Ogawa, H. Protease inhibitor therapy for recessive dystrophic epidermolysis bullosa. *In vitro* effect and clinical trial with camostat mesylate. *Journal of the American Academy of Dermatology* 18:1246-1252, 1988.

Ikeda, S., Naito, K., Imai, R., Manabe, M., Takamori, K. and Ogawa, H. Origin and properties of the blister formation factor in blister fluids from recessive dystrophic epidermolysis bullosa. *British Journal of Dermatology* 113:661-667, 1985.

Inaba, Y., Kitamura, K., Ogawa, H., Manabe, M. and Sasai, Y. A study on the estimation of prevalence of epidermolysis bullosa in Japan. *Nippon Hifuka Gakkai Zasshi - Japanese Journal of Dermatology* 99:1021-1026, 1989.

Ishida-Yamamoto, A., McGrath, J.A., Chapman, S.J., Leigh, I.M., Lane, E.B. and Eady, R.A. Epidermolysis bullosa simplex (Dowling-Meara type) is a genetic disease characterized by an abnormal keratin-filament network involving keratins K5 and K14. *Journal of Investigative Dermatology* 97:959-968, 1991.

Ito, M., Okuda, C., Shimizu, N., Tazawa, T. and Sato, Y. Epidermolysis bullosa simplex (Koebner) is a keratin disorder. Ultrastructural and immunohistochemical study. *Archives of Dermatology* 127:367-372, 1991.

Janmey, P.A., Euteneuer, U., Traub, P. and Schliwa, M. Viscoelastic properties of vimentin compared with other filamentous biopolymer networks. *Journal of Cell Biology* 113:155-160, 1991.

Jennings, J.L. Aluminum chloride hexahydrate treatment of localized epidermolysis bullosa. *Archives of Dermatology* 120:1382, 1984.

Kanzler, M.H., Smoller, B. and Woodley, D.T. Congenital localized absence of the skin as a manifestation of epidermolysis bullosa. *Archives of Dermatology* 128:1087-1090, 1992.

Kates, S.G., Sueki, H., Honig, P.J. and Murphy, G.F. Immunohistochemical and ultrastructural characterization of tonofilament and hemidesmosome abnormalities in a case of epidermolysis bullosa herpetiformis (Dowling-Meara). *Journal of the American Academy of Dermatology* 27:929-934, 1992.

Kennedy, A.R., Heagerty, A.H., Ortonne, J.P., Hsi, B.L., Yeh, C.J. and Eady, R.A. Abnormal binding of an anti-amnion antibody to epidermal basement membrane provides a novel diagnostic probe for junctional epidermolysis bullosa. *British Journal of Dermatology* 113:651-659, 1985.

Kero, M. Occurrence of epidermolysis bullosa in Finland. *Acta Dermato-Venereologica* 64:57-62, 1984.

Kero, M. and Niemi, K.M. Epidermolysis bullosa. *International Journal of Dermatology* 25:75-82, 1986.

Kessler, D.A., Siegel, J.P., Noguchi, P.D., Zoon, K.C., Feiden, K.L. and Woodcock, J. Regulation of somatic-cell therapy and gene therapy by the food and drug administration. *The New England Journal of Medicine* 329:1169-1173, 1993.

Kindler, T. Congenital poikloderma with traumatic bullae formation and progressive cutaneous atrophy. *British Journal of Dermatology* 66:104-111, 1954.

Kitajima, Y., Inoue, S. and Yaoita, H. Abnormal organization of keratin intermediate filaments in cultured keratinocytes of epidermolysis bullosa simplex. *Archives of Dermatological Research* 281:5-10, 1989.

- Kletter, G., Evans, O.B., Lee, J.A., Melvin, B., Yates, A.B. and Bock, H.G. Congenital muscular dystrophy and epidermolysis bullosa simplex. *Journal of Pediatrics* 114:104-107, 1989.
- Klymkowsky, M.W., Miller, R.H. and Lane, E.B. Morphology, behavior, and interaction of cultured epithelial cells after antibody-induced disruption of keratin filament organization. *Journal of Cell Biology* 96:494-509, 1983.
- Knapp, A.C., Franke, W.W., Heid, H., Hatzfeld, M., Jorcano, J.L. and Moll, R. Cytokeratin no. 9, an epidermal type I keratin characteristic of a special program of keratinocyte differentiation displaying body site specificity. *Journal of Cell Biology* 103:657-667, 1986.
- Koebner, H. Hereditäre Anlage Zur Blasenbildung (Epidermolysis bullosa hereditaria). *Dtsch Med. Wochenschr* 12:21-22, 1886.
- Kohn, C.W., Johnson, G.C., Garry, F., Johnson, C.W., Martin, S. and Scott, D.W. Mechanobullous disease in two Belgium foals. *Equine Veterinary Journal* 21:297-301, 1989.
- Korge, B.P., Compton, J.G., Steinert, P.M. and Mischke, D. The two size alleles of human keratin 1 are due to a deletion in the glycine-rich carboxyl-terminal V2 subdomain. *Journal of Investigative Dermatology* 99:667-702, 1992a.
- Korge, B.P., Gan, S.Q., McBride, O.W., Mischke, D. and Steinert, P.M. Extensive size polymorphism of the human keratin 10 chain resides in the C-terminal V2 subdomain due to variable numbers and sizes of glycine loops. *Proceedings of the National Academy of Sciences of the United States of America* 89:910-914, 1992b.
- Kouklis, P.D., Papamarcaki, T., Merdes, A. and Georgatos, S.D. A potential role for the COOH-terminal domain in the lateral packing of type III intermediate filaments. *Journal of Cell Biology* 114:773-786, 1991.
- Krieg, T.M., Schafer, M.P., Cheng, C.K., Filpula, D., Flaherty, P., Steinert, P.M. and Roop, D.R. Organization of a type I keratin gene. Evidence for evolution of intermediate filaments from a common ancestral gene. *Journal of Biological Chemistry* 260:5867-5870, 1985.
- Kulesh, D.A., & Oshima, R.G. Cloning of the human keratin 18 gene and its expression in non-epithelial mouse cells. *Molecular and Cellular Biology* 8:1540-1550, 1988.
- Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685, 1970.
- Lane, E.B. Monoclonal antibodies provide specific intramolecular markers for the study of epithelial tonofilament organization. *Journal of Cell Biology* 92:665-673, 1982.
- Lane, E.B. and Alexander, C.M. Use of keratin antibodies in tumor diagnosis. *Seminars in Cancer Biology* 1:165-179, 1990.
- Lane, E.B., Bartek, J., Purkis, P.E. and Leigh, I.M. Keratin antigens in differentiating skin. *Journal of Cell Biology* 95:580-588, 1985.

Lane, E.B., Rugg, E.L., Navsaria, H., Leigh, I.M., Heagerty, A.H., Ishida-Yamamoto, A. and Eady, R.A. A mutation in the conserved helix termination peptide of keratin 5 in hereditary skin blistering. *Nature* 356:244-246, 1992.

Lees, J.F., Shneidman, P.S., Skunzt, S.F., Carden, M.J. and Lazzarini, R.A. The structure and organization of the human heavy neurofilament subunit (NF-H) and the gene encoding it. *Embo Journal* 7:1974-1955, 1988.

Leigh, I.M., Tidman, M.J. and Eady, R.A. Epidermolysis bullosa: preliminary observations of blister formation in keratinocyte cultures. *British Journal of Dermatology* 111:527-532, 1984.

Lersch, R. and Fuchs, E. Sequence and expression of a type II keratin, K5, in human epidermal cells. *Molecular and Cellular Biology* 8:486-493, 1988.

Lersch, R., Stellmach, V., Stocks, C., Giudice, G. and Fuchs, E. Isolation, sequence, and expression of a human keratin K5 gene: transcriptional regulation of keratins and insights into pairwise control. *Molecular and Cellular Biology* 9:3685-3697, 1989.

Lessin, S.R., Huebner, K., Isobe, M., Croce, C.M. and Steinert, P.M. Chromosomal mapping of human keratin genes: evidence of nonlinkage. *Journal of Investigative Dermatology* 91:572-579, 1988.

Letai, A., Coulombe, P.A. and Fuchs, E. Do the ends justify the mean? Proline mutations at the ends of the keratin coiled-coil rod segment are more disruptive than internal mutations. *Journal of Cell Biology* 116:1181-1195, 1992.

Letai, A., Coulombe, P.A., McCormick, M.B., Yu, Q.C., Hutton, E. and Fuchs, E. Disease severity correlates with position of keratin point mutations in patients with epidermolysis bullosa simplex. *Proceedings of the National Academy of Sciences of the United States of America* 90:3197-3201, 1993.

Lewin, B., *Genes II*. John Wiley & Sons. 341, 1985.

Lieberburg, I., Spinner, N., Snyder, S., Anderson, J., Goldgaber, D., Smulowitz, M., Carroll, Z., Emanuel, B., Breitner, J. and Rubin, L. Cloning of a cDNA encoding the rat high molecular weight neurofilament peptide (NF-H): development and tissue expression in the rat and mapping of its human homologue to chromosomes 1 and 22. *Proceedings of the National Academy of Sciences of the United States of America* 86:2463-2467, 1989.

Lin, A.N. and Carter, D.M. Epidermolysis bullosa: when the skin falls apart. *Journal of Pediatrics* 114:349-355, 1989.

Lin, A.N. and Carter, D.M. *Epidermolysis Bullosa: Basic and Clinical Aspects*, eds. Lin, A.N. and Carter, D.M. Springer-Verlag, New York, Inc. vii, 1992a.

Lin, A.N. and Carter, D.M. Epidermolysis bullosa: a clinical overview. *Epidermolysis Bullosa: Basic and Clinical Aspects*, eds. Lin, A.N. and Carter, D.M. Springer-Verlag, New York, Inc. 89-117, 1992b.

Lofberg, L., Anton-Lamprecht, I., Michaelsson, G. and Gustavii, B. Prenatal exclusion of Herlitz syndrome by electron microscopy of fetal skin biopsies obtained at fetoscopy. *Acta Dermato-Venereologica* 63:185-189, 1983.

- Lu, X. and Lane, E.B. Retrovirus-mediated transgenic keratin expression in cultured fibroblasts: specific domain functions in keratin stabilization and filament formation. *Cell* 62:681-696, 1990.
- Lunstrum, G.P., Sakai, L.Y., Keene, D.R., Morris, N.P. and Burgeson, R.E. Large complex globular domains of type VII procollagen contribute to the structure of anchoring fibrils. *Journal of Biological Chemistry* 261:9042-9048, 1986.
- McConnell, S.J. and Yaffe, M.P. Intermediate filament formation by a yeast protein essential for organelle inheritance. *Science* 260:687-689, 1993.
- McCormick, M.B., Coulombe, P.A. and Fuchs, E. Sorting out IF networks: consequences of domain swapping on IF recognition and assembly. *Journal of Cell Biology* 113:1111-1124, 1991.
- McGrath, J.A., Hartley, M. and Eady, R.A.J. Epidermolysis bullosa in the United Kingdom: establishing a national register (abstract). *British Journal of Dermatology* 127, suppl. 40:29 1992a.
- McGrath, J.A., Ishida-Yamamoto, A., Tidman, M.J., Heagerty, A.H., Schofield, O.M. and Eady, R.A. Epidermolysis bullosa simplex (Dowling-Meara). A clinicopathological review. *British Journal of Dermatology* 126:421-430, 1992b.
- McKenna, K.E., Hughes, A.E., Bingham, E.A. and Nevin, N.C. Linkage of epidermolysis bullosa simplex to keratin gene loci. *Journal of Medical Genetics* 29:568-570, 1992a.
- McKenna, K.E., Walsh, M.Y. and Bingham, E.A. Epidermolysis bullosa in Northern Ireland. *British Journal of Dermatology* 127:318-321, 1992b.
- McKinley-Grant, L.J., Idler, W.W., Bernstein, I.A., Parry, D.A., Cannizzaro, L., Croce, C.M., Huebner, K., Lessin, S.R. and Steinert, P.M. Characterization of a cDNA clone encoding human filaggrin and localisation of the gene to chromosome region 1q21. *Proceedings of the National Academy of Sciences of the United States of America* 86:4848-4852, 1989.
- McLean, W.H. and Lane, E.B. A₁ RFLP of human keratin 10 (KRT-10) detected by PCR. *Human Molecular Genetics* 1:659, 1992.
- McLean, W.H.I., Eady, R.A., Dopping-Hepenstal, P.J.C., McMillan, J.R., Leigh, I.M., Navsaria, H.A., Higgins, C., Harper, J.I., Paige, D.G., Morley, S.M. and Lane, E.B. Mutations in the rod 1A domain of keratins 1 and 10 in bullous congenital ichthyosiform erythrodermal (BCIE). *Journal of Investigative Dermatology* 102:24-30, 1994.
- McLean, W.H., Morley, S.M., Lane, E.B., Eady, R.A., Griffith, W.A., Paige, D.G., Harper, J.I., Higgins, C. and Leigh, I.M. Ichthyosis Bullosa of Siemens - a disease involving keratin 2e. *Journal of Investigative Dermatology* 103:277-281, 1994.
- Manabe, M., Naito, K., Ikeda, S., Takamori, K. and Ogawa, H. Production of blister in normal human skin *in vitro* by blister fluids from epidermolysis bullosa. *Journal of Investigative Dermatology* 82:283-286, 1984.
- Marchuk, D., McCrohn, S. and Fuchs, E. Remarkable conservation of structure among intermediate filament genes. *Cell* 39:491-498, 1984.

- Marchuk, D., McCrohn, S. and Fuchs, E. Complete sequence of a gene encoding a human type I keratin: sequence homologous to enhancer elements in the regulatory region of the gene. *Proceedings of the National Academy of Sciences of the United States of America* 82:1609-1613, 1985.
- Marinkovich, M.P., Verrando, P., Keene, D.R., Meneguzzi, G., Lunstrum, G.P., Ortonne, J.P. and Burgeson, R.E. Basement membrane proteins kalinin and nicein are structurally and immunologically identical. *Laboratory Investigation* 69:295-299, 1993.
- Matsumoto, M. and Hashimoto, K. Blister fluid from epidermolysis bullosa letalis induces dermal-epidermal separation at lamina lucida (abstract). *Journal of Investigative Dermatology* 82:392-393, 1984.
- Matsumoto, M. and Hashimoto, K. Blister fluid from epidermolysis bullosa letalis induces dermal-epidermal separation *in vitro*. *Journal of Investigative Dermatology* 87:117-121, 1986.
- Matsumoto, M., Hashimoto, K. and Ohkawara, A. The role of lysosomal protease in junctional blister formation in epidermolysis bullosa letalis (abstract). *Journal of Investigative Dermatology* 84:354, 1985.
- Matsumura, K.K., Bonifas, J.M., Bare, J.W., Chen, M.A., Berth-Jones, J., Zloczower, M., Fritsch, P.O. and Epstein, E.H., Jr. Linkage to the type I keratin gene cluster of palmoplantar epidermolytic hyperkeratosis (abstract). *Journal of Investigative Dermatology* 100:508, 1993.
- Medenica-Mojilovic, L., Fenske, N.A. and Espinoza, C.G. Epidermolysis bullosa herpetiformis with mottled pigmentation and an unusual punctate keratoderma. *Archives of Dermatology* 122:900-908, 1986.
- Mendes da Costa, S. and van der Valk, J.W. Typhus maculatus der bullosen hereditaren dystrophie. *Archives of Dermatology Syph* 91:1, 1908.
- Miller, A.D. Progress toward human gene therapy. *Blood* 76:271-278, 1990.
- Miller, A.D. Human gene therapy comes of age. *Nature* 357:455-460, 1992.
- Miller, R.K., Khuon, S. and Goldman, R.D. Dynamics of keratin assembly: exogenous type I keratin rapidly associates with type II keratin *in vivo*. *Journal of Cell Biology* 122:123-135, 1993.
- Miller, R.K., Vikstrom, K. and Goldman, R.D. Keratin incorporation into intermediate filament networks is a rapid process. *Journal of Cell Biology* 113:843-855, 1991.
- Mischke, D. and Wild, G. Polymorphic keratins in human epidermis. *Journal of Investigative Dermatology* 88:191-197, 1987.
- Mischke, D., Wild, G. and Wild, A.G. Allele frequencies and segregation of human polymorphic keratins K4 and K5. *American Journal of Human Genetics* 46:548-552, 1990.
- Mittal, B., Sanger, J.M. and Sanger, J.W. Visualization of intermediate filaments in living cells using fluorescently labeled desmin. *Cell Motility & the Cytoskeleton* 12:127-138, 1989.

- Moll, I., Heid, H., Franke, W.W., and Moll, R. Distribution of a special subset of keratinocytes characterized by the expression of cytokeratin 9 in adult and fetal human epidermis of various body sites. *Differentiation* 33:254-265, 1987.
- Moll, R., Dhouailly, D. and Sun, T-T. Expression of keratin 5 as a distinctive feature of epithelial and biphasic mesotheliomas - an immunohistochemical study using monoclonal antibody AE14. *Vichows Arch.* 58:105-113, 1988.
- Moll, R., Franke, W.W., Schiller, D.L., Geiger, B. and Krepler, R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 31:11-24, 1982.
- Moll, R., Schiller, D.L. and Franke, W.W. Identification of protein IT of the intestinal cytoskeleton as a novel type I cytokeratin with unusual properties and expression patterns. *Journal of Cell Biology* 111:567-580, 1990.
- Morioka, S., Naito, K. and Ogawa, H. The pathogenic role of pemphigus antibodies and proteinase in epidermal acantholysis. *Journal of Investigative Dermatology* 76:337-341, 1981.
- Mulley, J.C., Nicholls, C.M., Probert, D.N., Turner, T. and Sutherland, G.R. Genetic linkage analysis of epidermolysis bullosa simplex, Koebner type. *American Journal of Medical Genetics* 19:573-577, 1984.
- Mulligan, R.C. The basic science of gene therapy. *Science* 260:926-932, 1993.
- Naito, K., Morioka, S. and Ogawa, H. The pathogenic mechanism of blister formation in bullous pemphigoid. *Journal of Investigative Dermatology* 79:303-306, 1982.
- Niemi, K.M., Kero, M., Kanerva, L. and Mattila, R. Epidermolysis bullosa simplex. A new histologic subgroup. *Archives of Dermatology* 119:138-141, 1983.
- Niemi, K.M., Sommer, H., Kero, M., Kanerva, L. and Haltia, M. Epidermolysis bullosa simplex associated with muscular dystrophy with recessive inheritance. *Archives of Dermatology* 124:551-554, 1988.
- Newport, J.W., Wilson, K.L. and Dunphy, W.G. A lamin-independent pathway for nuclear envelope assembly. *Journal of Cell Biology* 111:2247-2259, 1990.
- Noonberg, S.B., Garvoy, M.R. and Hunt, C.A. Characteristics of oligonucleotide uptake in human keratinocyte cultures. *Journal of Investigative Dermatology* 101:727-731, 1993.
- Olaisen, B. and Gedde-Dahl, T., Jr. GPT--epidermolysis bullosa simplex (EBS Ogna) linkage in man. *Human Heredity* 23:189-196, 1973.
- Oshima, R.G. Intermediate filament molecular biology. *Current Opinion in Cell Biology* 4:110-116, 1992.
- Ott, J., Estimation of the recombinant fraction in human pedigrees: efficient computation of the likelihood for human linkage studies. *American Journal of Human Genetics* 26:588-597, 1974.

- Parry, D.A. Primary and secondary structure of intermediate filament protein chains and modes of molecular aggregation. *Cellular and Molecular Biology of Intermediate Filaments*, eds. Goldman, R.D. and Steinert, P.M. Plenum Press Corp. New York. 175-197, 1990.
- Parry, D.A., Conway, J.F. and Steinert, P.M. Structural studies on lamin: similarities and differences between lamin and intermediate filament proteins. *Biochemical Journal* 238:305-308, 1986.
- Pavicic, Z., Kmet-Vizintin, P., Kansky, A. and Dobric, I. Occurrence of hereditary bullous epidermolyses in Croatia. *Pediatric Dermatology* 7:108-110, 1990.
- Pearson, R.W. Studies on the pathogenesis of epidermolysis bullosa. *Journal of Investigative Dermatology* 39:551-575, 1962.
- Pearson, R.W. The mechanobullous diseases (epidermolysis bullosa). *Dermatology in General Medicine, 1st Ed*, eds. Fitzpatrick, T.B., Arndt, K.A., Clark, W.H., Eisen, A.Z., van Scott, E.J. and Vaughan, J.H. McGraw Hill, New York. 621-647, 1971.
- Pearson, R.W. Clinicopathologic types of epidermolysis bullosa and their nondermatological complications. *Archives of Dermatology* 124:718-725, 1988.
- Peter, M., Heitlinger, E., Haner, M., Aebi, U. and Nigg, E.A. Disassembly of *in vitro* formed lamin head-to-tail polymers by cdc2 kinase. *Embo Journal* 10:1535-1544, 1991.
- Phillips, S.B. and Baden, H.P. Ichthyosiform dermatoses. *Dermatology in General Medicine I, 4th Ed*, eds. Fitzpatrick, T.B., Eisen, A.Z., Wolff, K., Freedberg, I.M. and Austen, K.F. McGraw Hill, Inc. 531-544, 1993.
- Pillai, S., Bikle, D.D., Mancianti, M.L., Cline, P. and Hincenbergs, M. Calcium regulation of growth and differentiation of normal human keratinocytes: modulation of differentiation competence by stages of growth and extracellular calcium. *Journal of Cellular Physiology* 143:294-302, 1990.
- Pittelkow, M.R. and Scott, R.E. New techniques for the *in vitro* culture of human skin keratinocytes and perspectives on their use for grafting of patients with extensive burns. *Mayo Clinic Proceedings* 61:771-777, 1986.
- Polakowska, R.R., Eddy, R.L., Shows, T.B. and Goldsmith, L.A. Epidermal type I transglutaminase (TGM1) is assigned to human chromosome 14. *Cytogenetics & Cell Genetics* 56:105-107, 1991.
- Popescu, N.C., Bowden, P.E. and Dipaolo, J.A. Two type II keratin genes are localized on chromosome 12. *Human Genetics* 82:109-112, 1989.
- Powell, B.C. and Rogers, G.E. Cyclic hair-loss and regrowth in transgenic mice overexpressing an intermediate filament gene. *Embo Journal* 9:1485-1493, 1990.
- Priestley, G.P. An introduction to skin and its diseases. *Molecular Aspects of Dermatology*, ed. Priestley, G.P. John Wiley & Sons, Chichester, England. 1-19, 1993.
- Pruss, R.M., Mirsky, R., Raff, M.C., Thorpe, R., Dowding, A.J. and Anderton, B.H. All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. *Cell* 27:419-428, 1981.

- Pulkkinen, L., Christiano, A.M., Airenne, T., Haakana, H., Tryggvason, K. and Uitto, J. Mutations in the gamma 2 chain gene (LAMC2) of kalinin/laminin 5 in the junctional forms of epidermolysis bullosa. *Nature Genetics* 6:239-237, 1994.
- Purkis, P. E., Steel, J.B., Mackenzie, I.C., Nathrath, W.B.J., Leigh, I.M. and Lane E.B. Antibody markers of basal cells in complex epithelia. *Journal of Cell Science* 97:39-50, 1990.
- Qian, F., Bajkowski, A.S., Steiner, D.F., Chan, S.J. and Frankfater, A. Expression of five cathepsins in murine melanomas of varying metastatic potential and normal tissues. *Cancer Research* 49:4870-4875, 1989.
- Quax, W., Khan, P.M., Quax-Jeuken, Y. and Bloemendal, H. The human desmin and vimentin genes are located on different chromosomes. *Gene* 38:189-196, 1985.
- Ramaekers, F.C., Huysmans, A., Schaart, G., Mosker, O. and Vooijs, P. Tissue distribution of keratin 7 as monitored by a monoclonal antibody. *Experimental Cell Research* 170:235-249, 1987.
- Raychaudhury, A., Marchuk, D., Lindhurst, M. and Fuchs, E. Three tightly linked genes encoding human type I keratins: conservation of sequence in the 5'-untranslated leader and 5'-upstream regions of coexpressed keratin genes. *Molecular and Cellular Biology* 6:539-548, 1986.
- Reis, A., Hennies, H., Langbein, L., Digweed, M., Mischke, D., Drechsler, M., Schrock, E., Royer-Pokora, B., Franke, W.W., Sperling, K. and Kuster, W. Keratin 9 gene mutations in epidermolytic palmoplantar keratoderma (EPPK). *Nature Genetics* 6:174-179, 1994.
- Reis, A., Kuster, W., Eckardt, R. and Sperling, K. Mapping of a gene for epidermolytic palmoplantar keratoderma to the region of the acidic keratin gene cluster at 17q12-q21. *Human Genetics* 90:113-116, 1992.
- Rheinwald, J.G. and Green, H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331-343, 1975.
- Rodeck, C.H., Eady, R.A. and Gosden, C.M. Prenatal diagnosis of epidermolysis bullosa letalis. *Lancet* 1:949-952, 1980.
- Romano, V., Bosco, P., Rocchi, M., Costa, G., Leube, R.E., Franke, W.W. and Romeo, G. Chromosomal assignments of human type I and type II cytokeratin genes to different chromosomes. *Cytogenetics & Cell Genetics* 48:145-151, 1988.
- Rosenberg, M., Fuchs, E., Le Beau, M.M., Eddy, R.L. and Shows, T.B. Three epidermal and one simple epithelial type II keratin genes map to human chromosome 12. *Cytogenetics & Cell Genetics* 57:33-38, 1991.
- Rosenberg, M., Raychaudhury, A., Shows, T., LeBeau, M.M. and Fuchs, E. A group of type I keratin genes on human chromosome 17: characterization and expression. *Molecular and Cellular Biology* 8:722-736, 1988.
- Rothnagel, J.A., Dominey, A.M., Dempsey, L.D., Longley, M.A., Greenhalgh, D.A., Gagne, T.A., Huber, M., Frenk, E., Hohl, D. and Roop, D.R. Mutations in the rod domains of keratins 1 and 10 in epidermolytic hyperkeratosis. *Science* 257:1128-1130, 1992.

Rothnagel, J.A., Fisher, M.P., Axtell, S.M., Pittelkow, M.R., Anton-Lamprecht, L., Huber, M., Hohl, D. and Roop, D.R. A mutational hot spot in keratin 10 (KRT 10) in patients with epidermolytic hyperkeratosis. *Human Molecular Genetics* 12:2147-2150, 1993.

Rothnagel, J.A., Longley, M.A., Holder, R.A., Kuster, W. and Roop, D.R. Prenatal diagnosis of epidermolytic hyperkeratosis by direct gene sequencing. *Journal of Investigative Dermatology* 102:13-16, 1994.

Rousselle, P., Lunstrum, G.P., Keene, D.R. and Burgeson, R.E. Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *Journal of Cell Biology* 114:567-571, 1991

Rugg, E.L. Detection and characterization of keratins by immunocytochemistry and immunoblotting. *Keratinocyte Methods*, eds. Leigh, I.M., Lane, E.B. and Watt, F. Cambridge University Press. 125-146, 1994.

Rugg, E.L., Morley, S.M., Smith, F.J.D., Boxer, M., Tidman, M.J., Navsaria, H., Leigh, I.M. and Lane, E.B. Missing links: Weber-Cockayne keratin mutations implicate the L12 linker domain in effective cytoskeleton function. *Nature Genetics* 5:294-300, 1993a.

Rugg, E.L., Smith, F.J.D., Navsaria, H., Morley, S.M., Wojnarowska, F., Leigh, I.M. and Lane, E.B. Skin fragility in Weber-Cockayne EBS caused by mutations in epidermal keratins (abstract). *British Journal of Dermatology* 129:486, 1993b.

Rupniak, H.T., Rowlatt, C., Lane, E.B., Steele, J.G., Trejdosiewicz, L. K., Laskiewicz, B., Povey, S. and Hill, B.T. Characteristics of four new human cell lines derived from squamous cell carcinomas of the head and neck. *Journal of the National Cancer Institute* 75:621-635, 1985.

Ryynanen, M., Knowlton, R.G., Parente, M.G., Chung, L.C., Chu, M.L. and Uitto, J. Human type VII collagen: genetic linkage of the gene (COL7A1) on chromosome 3 to dominant dystrophic epidermolysis bullosa. *American Journal of Human Genetics* 49:797-803, 1991a.

Ryynanen, M., Knowlton, R.G. and Uitto, J. Mapping of epidermolysis bullosa simplex mutation to chromosome 12. *American Journal of Human Genetics* 49:978-984, 1991b.

Ryynanen, M., Ryynanen, J., Sollberg, S., Iozzo, R.V., Knowlton, R.G. and Uitto, J. Genetic linkage of type VII collagen (COL7A1) to dominant dystrophic epidermolysis bullosa in families with abnormal anchoring fibrils. *Journal of Clinical Investigation* 89:974-980, 1992.

Sagi, A., Stahl, N. and Hauben, D. Omiderm: a synthetic cover for exposed areas in epidermolysis bullosa. *Annals of Plastic Surgery* 20:133-135, 1988.

Saiki, R.H. *PCR technology: principles and applications*, ed. Erlich, H.E. Stockton Press, New York. 1989.

St.Louis, D. and Verma, I.M. An alternative approach to somatic cell gene therapy. *Proceedings of the National Academy of Sciences of the United States of America* 85:3150-3154, 1988.

Sakai, L.Y., Keene, D.R., Morris, N.P. and Burgeson, R.E. Type VII collagen is a major structural component of anchoring fibrils. *Journal of Cell Biology* 103:1577-1586, 1986.

Sakamoto, F., Hachisuka, H., Nomura, H. and Sasai, Y. Abnormal binding of lectin to the epidermal cell membranes in the skin of epidermolysis bullosa simplex. *Acta Histochemica* 84:93-99, 1988.

Salih, M.A., Lake, B.D., el Hag, M.A. and Atherton, D.J. Lethal epidermolytic epidermolysis bullosa: a new autosomal recessive type of epidermolysis bullosa. *British Journal of Dermatology* 113:135-143, 1985.

Sambrook, J., Fritsh, E.F. and Maniatis, T. *Molecular cloning - a Laboratory Manual*. Cold Spring Harbour Laboratory, New York. 1989.

Sanchez, G., Seltzer, J.L., Eisen, A.Z., Stapler, P. and Bauer, E.A. Generalized dominant epidermolysis bullosa simplex: decreased activity of a gelatinolytic protease in cultured fibroblasts as a phenotypic marker. *Journal of Investigative Dermatology* 81:576-579, 1983.

Sanger, F. Nicklen, S. and Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 74:5463-5467, 1977.

Sarkany, I., Grice, K. and Caron, G.A. Organ culture of adult human skin. *British Journal of Dermatology* 77:65-76, 1965.

Savolainen, E.R., Kero, M., Pihlajaniemi, T. and Kivirikko, K.I. Deficiency of galactosylhydroxylsyl glucosyltransferase, an enzyme of collagen synthesis, in a family with dominant epidermolysis bullosa simplex. *New England Journal of Medicine* 304:197-204, 1981.

Savtchenko, E.S., Freedberg, I.M., Choi, I.Y. and Blumenberg, M. Inactivation of human keratin genes: the spectrum of mutations in the sequence of an acidic keratin pseudogene. *Molecular Biology and Evolution* 5:97-108, 1988a.

Savtchenko, E.S., Schiff T.A., Jiang, C.K., Freedberg, I.M. and Blumenberg, M. Embryonic expression of the human 40-kD keratin: evidence from a processed pseudogene sequence. *American Journal of Human Genetics* 43:630-637, 1988b.

Schiltz, J.R., Michel, B. and Papay, R. Appearance of "pemphigus acantholysis factor" in human skin cultured with pemphigus antibody. *Journal of Investigative Dermatology* 73:575-581, 1979.

Schofield, O.M., Fine, J.D., Verrando, P., Heagerty, A.H., Ortonne, J.P. and Eady, R.A. GB3 monoclonal antibody for the diagnosis of junctional epidermolysis bullosa: results of a multicenter study. *Journal of the American Academy of Dermatology* 23:1078-1083, 1990.

Scott, D.W. and Schultz, R.D. Epidermolysis bullosa simplex in the Collie dog. *Journal of the American Veterinary Medical Association* 171:721-727, 1977.

Seo, J. and Cohen, C. Pitch Diversity in α -helical coiled coils. *Proteins:Structure, Function and Genetics* 15:223-234, 1993.

Skoven, I. and Drzewiecki, K.T. Congenital localized skin defect and epidermolysis bullosa hereditaria letalis. *Acta Dermato-Venerologica* 59:533-537, 1979.

Smith, P.K., Davidson, G.P., Moore, L., Clapton, W., Jaunzems, A., Little, K.E. and Moore, D.J. Epidermolysis bullosa and severe ulcerative colitis in an infant. *Journal of Pediatrics* 122:600-603, 1993.

Smith, S.Z. and Cram, D.L. A mechanobullous disease of the newborn. Bart's syndrome. *Archives of Dermatology* 114:81-84, 1978.

Sommer, S.S., Groszbach, A.R. and Bottema, C.D. PCR amplification of specific alleles (PASA) is a general method for rapidly detecting known single-base changes. *Biotechniques* 12:82-87, 1992.

Sonnenberg, A., Calafat, J., Janssen, H., Daams, H., van der Raaij-Helmer, L.M.H., Falcioni, R., Kennel, S.J., Alpin, J.D., Baker, J., Loizidou, M. and Garrod, D. Integrin $\alpha 6 \beta 4$ complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion. *Journal of Cell Biology* 113:907-917, 1991.

Stappenbeck, T.S. and Green, K.J. The desmoplakin carboxyl terminus coaligns with and specifically disrupts intermediate filament networks when expressed in cultured cells. *Journal of Cell Biology* 116:1197-1209, 1992.

Stasiak, P.C., Purkis, P.E., Leigh, I.M. and Lane, E.B. Keratin 19: predicted amino acid sequence and broad tissue distribution suggest it evolved from keratinocyte keratins. *Journal of Investigative Dermatology* 92:707-716, 1989.

Steinert, P.M. The dynamic phosphorylation of the human intermediate filament keratin 1 chain. *Journal of Biological Chemistry* 263:13333-13339, 1988.

Steinert, P.M. The two-chain coiled-coil molecule of native epidermal keratin intermediate filaments is a type I-type II heterodimer. *Journal of Biological Chemistry* 265:8766-8774, 1990.

Steinert, P.M. Structure, function, and dynamics of keratin intermediate filaments. *Journal of Investigative Dermatology* 100:729-734, 1993.

Steinert, P.M. and Freedberg, I.M. Molecular and cellular biology of keratins. *Physiology, Biochemistry and Molecular Biology of the Skin I, 2nd Ed*, ed. Goldsmith, L.A. Oxford University Press Inc. New York. 113-147, 1991.

Steinert, P.M. and Parry, D.A. Intermediate filaments: conformity and diversity of expression and structure. *Annual Review of Cell Biology* 1:41-65, 1985.

Steinert, P.M. and Parry, D.A. The conserved H1 domain of the type II keratin 1 chain plays an essential role in the alignment of nearest neighbor molecules in mouse and human keratin 1/keratin 10 intermediate filaments at the two- to four-molecule level of structure. *Journal of Biological Chemistry* 268:2878-2887, 1993.

Steinert, P.M., Idler, W.W., Aynardi-Whitman, M., Zackroff, R.V. and Goldman R.D. Heterogeneity of intermediate filaments assembled *in vitro*. *Cold Spring Harbour Symposium Quantitative Biology* 46:465-474, 1982.

Steinert, P.M., Marekov, L.N., Fraser, R.D. and Parry, D.A. Keratin intermediate filament structure: crosslinking studies yield quantitative information on molecular dimensions and mechanism of assembly. *Journal of Molecular Biology* 230:436-452, 1993.

Steinert, P.M., Parry, D.A., Racoosin, E.L., Idler, W.W., Steven, A.C., Trus, B.L. and Roop, D.R. The complete cDNA and deduced amino acid sequence of a type II mouse epidermal keratin of 60,000 Da: analysis of sequence differences between type I and type II keratins. *Proceedings of the National Academy of Sciences of the United States of America* 81:5709-5713, 1984.

Steinert, P.M., Rice, R.H., Roop, D.R., Trus, B.L. and Steven, A.C. Complete amino-acid sequence of a mouse epidermal keratin subunit and implications for the structure of intermediate filaments. *Nature* 302:794-800, 1983.

Steinert, P.M., Steven, A.C. and Roop, D.R. The molecular biology of intermediate filaments. *Cell* 42:411-420, 1985.

Stephens, K., Sybert, V.P., Wijsman, E.M., Ehrlich, P. and Spencer, A. A keratin 14 mutational hot spot for epidermolysis bullosa simplex, Dowling-Meara: implications for diagnosis. *Journal of Investigative Dermatology* 101:240-243, 1993.

Stepp, M.A., Spurr-Michaud, S., Tisdale, A., Elwell, J. and Gipson, I.K. $\alpha 6\beta 4$ integrin heterodimer is a component of hemidesmosomes. *Proceedings of the National Academy of Sciences of the United States of America* 87:8970-8974, 1990.

Steven, A.C., Hainfeld, J.F., Trus, B.L., Wall, J.S. and Steinert, P.M. Epidermal keratin filaments assembled *in vitro* have masses-per-unit-length that scale according to average subunit mass: structural basis for homologous packing of subunits in intermediate filaments. *Journal of Cell Biology* 97:1939-1944, 1983.

Stewart, M. Intermediate filaments: structure, assembly and molecular interactions. *Current Opinion in Cell Biology* 2:91-100, 1990.

Stewart, M. Intermediate filament structure and assembly. *Current Opinion in Cell Biology* 5:3-11, 1993.

Sun, T.T. and Green, H. Keratin filaments of cultured human epidermal cells. Formation of intermolecular disulfide bonds during terminal differentiation. *Journal of Biological Chemistry* 253:2053-2060, 1978.

Sybert, V.P. and Holbrook, K.A. Prenatal diagnosis and genetic screening for epidermolysis bullosa. *Epidermolysis Bullosa: Basic and Clinical Aspects*, eds. Lin, A.N. and Carter, D.M. Springer-Verlag, New York, Inc. 235-251, 1992.

Tadini, G., Ermacora, E., Cambiaghi, S., Brusasco, A. and Cavalli, R. Positive response to 5HT-2 antagonists in a family affected by epidermolysis bullosa Dowling-Meara type (letter). *Dermatology* 186:80, 1993.

Tai, I.T. and Sun, A.M. Microencapsulation of recombinant cells: a new delivery system for gene therapy. *FASEB Journal* 7:1061-1069, 1993.

Takahasmi, T. and Tang, J. Cathepsin D from porcine and bovine spleen. *Methods in Enzymology*, ed. Lorand, L. Academic Press Inc. 80:565-581, 1981.

Takamori, K., Ikeda, S., Naito, K. and Ogawa, H. Proteases are responsible for blister formation in recessive dystrophic epidermolysis bullosa and epidermolysis bullosa simplex. *British Journal of Dermatology* 112:533-538, 1985.

Takamori, K., Naito, K. and Ogawa, H. Epidermolysis bullosa simplex blister fluid induces an intra-epidermal blister in cultured normal skin. *British Journal of Dermatology* 109:643-646, 1983.

Takamori, K., Yoshiike, T., Morioka, S. and Ogawa, H. The role of proteases in the pathogenesis of bullous dermatoses. *International Journal of Dermatology* 27:533-539, 1988.

Tidman, M.J. The epidermal basement membrane. *Molecular Aspects of Dermatology*, ed. Priestley, G.P. John Wiley & Sons, Chichester, England. 75-88, 1993.

Tidman, M.J., Eady, R.A., Leigh, I.M. and Macdonald, D.M. Keratin expression in epidermolysis bullosa simplex (Dowling-Meara). *Acta Dermato-Venereologica* 68:15-20, 1988.

Timpl, R., Dziadek, M., Fujiwara, W., Nowack, H. and Wick, H. Nidogen: a new self-aggregating basement membrane protein. *European Journal of Biochemistry* 137:455-465, 1983.

Tkach, J.R. Treatment of recurrent bullous eruption of the hands and feet (Weber-Cockayne disease) with topical aluminium chloride. *Journal of the American Academy of Dermatology* 6:1095-1096, 1982.

Torchard, D., Blanchet-Bardon, C., Serova, O., Langbein, L., Narod, S., Janin, N., Goguel, A.F., Berheim, A., Franke, W.W., Lenoir, G.M. and Feuteun, J. Epidermolytic palmoplantar keratoderma cosegregates with a keratin 9 mutation in a pedigree with breast and ovarian cancer. *Nature Genetics* 6:106-110, 1994.

Traub, P. and Vorgias, C.E. Involvement of the N-terminal polypeptide of vimentin in the formation of intermediate filaments. *Journal of Cell Science* 63:43-67, 1983.

Troyanovsky, S.M., Leube, R.E. and Franke, W.W. Characterization of the human gene encoding cytokeratin 17 and its expression pattern. *European Journal of Cell Biology* 59:127-137, 1992.

Tsao, M.C., Walthall, B.J. and Ham, R.G. Clonal growth of normal human epidermal keratinocytes in a defined medium. *Journal of Cellular Physiology* 110:219-229, 1982.

Tyner, A.L., Eichman, M.J. and Fuchs, E. The sequence of a type II keratin gene expressed in human skin: conservation of structure among all intermediate filament genes. *Proceedings of the National Academy of Sciences of the United States of America* 82:4683-4687, 1985.

Tyner, A.L. and Fuchs, E. Evidence for posttranscriptional regulation of the keratins expressed during hyperproliferation and malignant transformation in human epidermis. *Journal of Cell Biology* 103:1945-1955, 1986.

Vaidya, S., Tying, S.K., Feldkamp, M., Johnson, L.B. and Fine, J.D. HLA and epidermolysis bullosa: evidence for independent assortment of Weber-Cockayne subtype of epidermolysis bullosa and HLA complex. *Journal of Dermatological Science* 2:155-160, 1991.

Vassar, R., Coulombe, P.A., Degenstein, L., Albers, K. and Fuchs, E. Mutant keratin expression in transgenic mice causes marked abnormalities resembling a human genetic skin disease. *Cell* 64:365-380, 1991.

Venetianer, A., Schiller, D.L., Magin, T. and Franke, W.W. Cessation of cytokeratin expression in a rat hepatoma cell line lacking differentiated functions. *Nature* 305:730-733, 1983.

Verrando, P., Blanchet-Bardon, C., Pisani, A., Thomas, L., Cambazard, F., Eady, R.A., Schofield, O. and Ortonne, J.P. Monoclonal antibody GB3 defines a widespread defect of several basement membranes and a keratinocyte dysfunction in patients with lethal junctional epidermolysis bullosa. *Laboratory Investigation* 64:85-92, 1991.

Verrando, P., C., Pisani, A. and Ortonne, J.P. The new basement membrane antigen recognized by the monoclonal antibody GB3 is a large size glycoprotein: modulation of its expression by retinoic acid. *Biochemica Et Biophysica Acta* 942:45-56, 1988.

Verrando, P., Schofield, O., Ishida-Yamamoto, A., Aberdam, D., Partouche, O., Eady, R.A.J. and Ortonne, J.P. Nicein (BM-600) in junctional epidermolysis bullosa: polyclonal antibodies provide new clues for pathogenic role. *Journal of Investigative Dermatology* 101:738-743, 1993.

Vikstrom, K.L., Borisy, G.G. and Goldman, R.D. Dynamic aspects of intermediate filament networks in BHK-21 cells. *Proceedings of the National Academy of Sciences of the United States of America* 86:549-553, 1989.

Vikstrom, K.L., Lim, S.S., Goldman, R.D. and Borisy, G.G. Steady state dynamics of intermediate filament networks. *Journal of Cell Biology* 118:121-129, 1992.

Vogel, J.C. Keratinocyte gene therapy. *Archives of Dermatology* 129:1478-1483, 1993.

Wanner, R., Forster, H.H., Tilmans, I. and Mischke, D. Allelic variations of human keratins K4 and K5 provide polymorphic markers within the type II keratin gene cluster on chromosome 12. *Journal of Investigative Dermatology* 100:735-741, 1993.

Wasco, W., Peppercorn, J. and Tanzi, R.E. Search for the genes responsible for familial Alzheimer's disease. *Annals of the New York Academy of Sciences* 695:203-8, 1993.

Waseem, A., Alexander, C.M., Steel, J.B. and Lane, E.B. Embryonic simple epithelial keratins 8 and 18: chromosomal location emphasizes difference from other keratin pairs. *New Biologist* 2:464-478, 1990.

Watt, F.M., Matthey, D.L. and Garrod, D.R. Calcium-induced reorganization of desmosomal components in cultured human keratinocytes. *Journal of Cell Biology* 99:2211-2215, 1984.

Weber, F.P. Recurrent bullous eruption of the feet in a child. *Proceedings of the Royal Society of Medicine* 19:72, 1926.

Weber, K., Plessmann, U. and Ulrich, W. Cytoplasmic intermediate filament proteins of invertebrates are closer to nuclear lamins than are vertebrate intermediate filament proteins; sequence characterization of two muscle proteins of a nematode. *Embo Journal* 8:3221-3227, 1989.

- Wesner, G. So-called recurrent blister eruption of the foot; hot weather (Weber-Cockayne) (summer eruption of the feet), epidermolysis bullosa tarda (epidermolysis bullosa pedum et manuum destivalis); epidermolysis bullosa Weber-Cockayne. *Dermatol. Wochenschr* 136:1133-1137, 1957.
- Wilson, A.K., Coulombe, P.A. and Fuchs, E. The roles of K5 and K14 head, tail, and R/K L L E G E domains in keratin filament assembly *in vitro*. *Journal of Cell Biology* 119:401-414, 1992.
- Winberg, J.O. and Gedde-Dahl, T. Gelatinase expression in generalized epidermolysis bullosa simplex fibroblasts. *Journal of Investigative Dermatology* 87:326-329, 1986.
- Winberg, J.O. and Gedde-Dahl, T., Jr. Epidermolysis bullosa simplex: expression of gelatinase activity in cultured human skin fibroblasts. *Biochemical Genetics* 30:401-420, 1992.
- Winship, I.M. Epidermolysis bullosa in South Africa: formation of a national registry. *Epidermolysis bullosa: A Comprehensive Review of Classification Management and Laboratory Studies*, eds. Priestley, G.C., Tidman, M.J., Weiss, J.B. and Eady, R.A. Crowthorne, England: Dystrophic Epidermolysis Bullosa Research Association. 134-136, 1990.
- Winship, I.M. and Winship, W.S. Epidermolysis bullosa misdiagnosed as child abuse. A report of 3 cases. *South African Medical Journal* 73:369-370, 1988.
- Woerderman, M.J. Dystrophia bullosa hereditaria. Typhus maculatus. Proceedings of the second international congress of dermatology. *Acta Dermato-Venerologica* 678:111-116, 1957.
- Wojnarowska, F., Eady, R.A.J. and Wells, R.S. Dystrophic epidermolysis bullosa presenting with congenital localized absence of skin: report of four cases. *British Journal of Dermatology* 108:477-483, 1983.
- Woodcock-Mitchell, J., Eichner, R., Nelson, W.G. and Sun, T-T. Immunolocalization of keratin polypeptides in human epidermis using monoclonal antibodies. *Journal of Cell Biology* 95:580-588, 1982.
- Woodgett, J.R., Gould, K.L. and Hunter, T. Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. *European Journal of Biochemistry* 161:177-184, 1986.
- Woodley, D.T. and McNutt, S. The basement membrane zone at the dermal-epidermal junction of human skin. *Epidermolysis Bullosa: Basic and Clinical Aspects*, eds. Lin, A.N. and Carter, D.M. Springer-Verlag, New York, Inc. 19-36, 1992.
- Wright, J.T., Capps, J. and Johnson L.B. Oral and ultrastructural dental manifestations of epidermolysis bullosa (abstract). *Journal of Dental Research* 67:249, 1988.
- Xu, Z., Cork, L.C., Griffin, J.W. and Cleveland, D.W. Increased expression of neurofilament subunit NF-L produces morphological alterations that resemble the pathology of human motor neuron disease. *Cell* 73:23-33, 1993.
- Yang, J-M., Chipev, C.C., DiGiovanna, J.J., Bale, S.J., Marekov, L.N., Steinert, P.M. and Compton, J.G. Mutations in the H1 and 1A domains in the keratin 1 gene in epidermolytic hyperkeratosis. *Journal of Investigative Dermatology* 102:24-30, 1994.

Younger, I.R., Priestley, G.C. and Tidman, M.J. Aluminum chloride hexahydrate and blistering in epidermolysis bullosa simplex. *Journal of the American Academy of Dermatology* 23:930-931, 1990.

Zamansky, G.B., Nguyen, U. and Chou, I. An immunofluorescence study of the calcium-induced coordinated reorganization of microfilaments, keratin intermediate filaments, and microtubules in cultured human epidermal keratinocytes. *Journal of Investigative Dermatology* 97:985-994, 1991.

APPENDICES

Appendix I

Cell Culture

Materials

Swiss mouse 3T3 cells and Hoechst stain H33258 were supplied by Flow. Cholera toxin, hydrocortisone, adenine, 3,3', 5-tri-iodo-L-thyronine and insulin were obtained from Sigma, all other media, supplements and Nunc tissue culture flasks and chamber slides were purchased from GIBCO BRL Life Technologies. DAPI stain and BM Cyclin were purchased from Boehringer. Secondary antibody for staining cells by immunofluorescence, sheep-anti-mouse FITC conjugated was from Sigma; for primary antibodies see Appendix III.

Methods

Transport medium : DMEM medium-10% FCS containing 200 U/ml penicillin, 200 µg/ml streptomycin and 0.25 µg/ml fungizone Amphotericin B.

RM-medium

3 : 1 DMEM medium : HAMS F12 containing 10% FCS and the following mitogens.

Final concentration in medium

Hydrocortisone	0.4 µg/ml
Cholera toxin	10^{-10} M
Transferrin	5 µg/ml
3, 3', 5-Tri-iodo-L-thyronine	2×10^{-9} M
Adenine	1.8×10^{-4} M
Insulin	5 µg/ml

RM+medium

3 : 1 DMEM medium : HAMS F12 containing 10% FCS and the same mitogens as the RM-medium with the addition of epidermal growth factor (EGF) at a final concentration of 10 ng/ml.

Concentrated stocks of the additives were made up, filter sterilised and stored in small aliquots at -20°C.

Hydrocortisone: dissolved in 95% ethanol to 5 mg/ml and diluted to 40 µg/ml in Hanks balanced salt solution to give a 100 x stock solution.

Cholera toxin : dissolved in distilled water at 1 mg/ml and diluted in Hanks balanced salt solution to give a 100 x stock solution.

Transferrin/ 3, 3', 5-tri-iodo-L-thyronine: 100 mg of transferrin was dissolved in 12 ml PBS. 13.6 mg 3, 3', 5-tri-iodo-L-thyronine was dissolved in a small volume of 0.2 N NaOH and made up to 100 ml with distilled water, to give a stock solution of 2×10^{-4} . The transferrin (12 ml) was added to 7.8 ml of distilled water and 0.2 ml of 3, 3', 5-tri-iodo-L-thyronine stock solution was added; 0.1 ml of this mixture was added to 100 ml of medium to give 5 µg/ml transferrin and 2×10^{-9} 3,3', 5-tri-iodo-L-thyronine.

Adenine: A 100 x stock was prepared by dissolving 243 mg of adenine in a minimum volume of HCl (1N) and made up to 100 ml with distilled water.

Insulin: dissolved in distilled water to 5 mg/ml (1000 x stock).

Epidermal growth factor: dissolved at 1 µg/ml in Hanks balanced salt solution 100 x stock).

Appendix II

DNA Analysis

Materials

The Quick Prep Micro mRNA purification kit was purchased from Pharmacia and the dsDNA cycle sequencing kit from BRL Life Technologies. AMV Reverse transcriptase was supplied by Promega, Amplitaq Polymerase by Perkin Elmer and dNTPs by Pharmacia. *AvaII*, *BsaII* and *PstI* and the appropriate buffers were purchased from Biolabs and *AluI* and *MboII* and buffers from International Biotechnologies Inc. *MaeII* and buffer, RNasin, standard DNA VI molecular weight markers (154-2176 base pairs), and dithiothreitol were all supplied by Boehringer. Poly-Pak cartridges (Glen Research Corporation) and Cambio taq and buffer were from Cambio. SequaGel Concentrate, SequaGel Diluent, SequaGel Buffer were from National Diagnostics, $\gamma^{32}\text{P}$ ATP and $\alpha^{32}\text{P}$ ATP from Amersham International and the X-ray film from Fuji. All other chemicals were supplied by BDH.

PCR and DNA cycle sequencing reactions were carried out in either a Perkin Elmer or Hybaid Omnigene machine.

Buffers

PCR buffer x 20 concentration (0 mM MgCl_2): 1 M KCl, 200 mM Tris pH 8.3, 2% Triton x-100. Made up in DEPC water and used at this concentration for reverse transcription.

PCR buffer x 20 concentration (20 mM MgCl_2): 1 M KCl, 200 mM Tris pH 8.3, 2% Triton x-100, 20 mM MgCl_2 . Made up in DEPC water and used at this concentration for PCR reactions.

Tris-acetate/EDTA buffer (TAE) x 50 concentration

Tris-base	242 g
glacial acetic acid	57.1 ml
0.5 M EDTA pH 8.0	100 ml

Made up to 1 l in milli Q water and diluted 1:50 (x1 concentration, 40 mM Tris-base, 1 mM EDTA) for agarose gel electrophoresis.

Tris-borate/EDTA buffer (TBE) x 5 concentration

Tris-base	54 g
boric acid	27.5 g
0.5 M EDTA pH 8.0	20 ml

Made up to 1 l in milli Q water and diluted 1:10 (x 0.5 concentration, 45 mM Tris-borate, 1 mM EDTA) for agarose gel electrophoresis and 1:5 (x1 concentration, 90 mM Tris-borate, 2 mM EDTA) for sequencing gels.

TAE/TBE sample buffer : TAE/TBE buffer x 6 concentration, containing 30% glycerol and 0.25% bromophenol blue.

Tris/EDTA buffer (TE) x 10 concentration

1M Tris-HCl	10 ml
0.5 mM EDTA pH 8.0	2 ml

Made up to 1 l in milli Q water and diluted 1:10 for use.

Formamide-EDTA buffer

95% (v/v) formamide
10 mM EDTA pH 8.0
0.1% (w/v) bromophenol blue
0.1% (w/v) xylene blue

Sequencing gel mixture (100 ml)

	6%	4%
SequaGel Concentrate	24 ml	16 ml
SequaGel Diluent	66 ml	74 ml
SequaGel Buffer	10 ml	10 ml
TEMED	40 µl	40 µl
10% ammonium persulphate	800 µl	800 µl

PCR protocols

The primers used for reverse transcription were:

Keratin 5: 5' GCTCTTCCGGGAGGAGGAGGT 3' 1495-1515; EMBL accession number M19723

Keratin 14: 5' AGTGCTTGGGCAGGAGAGGGG 3';4788-4808; EMBL accession number J00124.

Keratin 5 or keratin 14 cDNA was amplified using the same primer as for reverse transcription (see above) with a second primer.

Keratin 5: 5' GGTGGTGGCTTTGGGCTCGGT 3',

Keratin 14: 5' GGGGGAGCCTATGGGTTGGGG 3',

Fragments of genomic DNA were amplified with various overlapping primers (see DNA sequencing primers).

PCR reactions (100 μ l total volume) were carried out with each tube containing the following:

sense primer (50 μ g/ml)	10 μ l
antisense primer (50 μ g/ml)	10 μ l
cDNA	5 μ l
PCR buffer x 20 concentration (20 mM MgCl ₂)	5 μ l
DMSO	10 μ l
20 mM dNTP	1 μ l
DEPC water	59 μ l
Amplitaq polymerase	0.3 μ l (1.67 units)

The samples were mixed, centrifuged for 10-15 sec and a drop of mineral oil added to each tube to prevent evaporation of samples. The cycling parameters were 94°C for 5 min, followed by 30 cycles of 30 sec at 94°C, 1 min at 60°C and 2 min at 72°C and 1 cycle of 5 min at 72°C. For amplification of genomic DNA, 1 μ l (approximately 1 μ g) was used per 100 μ l reaction and the volume of water increased to 63 μ l. PCR products were resolved on agarose gels (1.5%).

DNA Sequencing

The method for dsDNA cycle sequencing was as described for the kit (BRL Life Technologies). The primers used for DNA sequencing are listed below. The chosen primer was end labelled with γ^{32} P ATP, the volumes given are for a 36 μ l reaction volume for one DNA sample.

primer (7 ng/ μ l)	1 μ l
kinase buffer (x 5 concentration)	1 μ l
DEPC water	1 μ l
γ^{32} P ATP	1 μ l
T4 polynucleotide kinase	1 μ l

The primer was incubated for 30 min at 37°C followed by 5 min at 55 °C, cooled and added to the pre-reaction mixture.

Pre-reaction mixture:

DEPC water	21 µl
DNA (purified, 5 ng/ml)	5 µl
Taq sequencing buffer	4.5 µl
Taq polymerase	0.5 µl
primer	5 µl

After mixing, 8 µl was added to each of four microfuge tubes containing 2 µl of one of the dideoxy-termination mixtures (A, C, G or T). The samples were mixed, centrifuged for 10 sec and incubated under the following conditions: 20 cycles of 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C followed by 10 cycles of 30 sec at 94°C and 1 min at 72°C. To each tube, 5 µl of formamide-EDTA buffer was added, the samples heated to 90°C for 5 min and the 5 µl loaded onto a 6% polyacrylamide /TBE sequencing gel.

The following primers were used for DNA sequencing:

Keratin 5:

sense	5' GGTGGTGGCTTTGGGCTCGGT 3'
	5' GTGGAGCTGGAGGCCAAGGTT 3'
	5' CGCATGTCTCTGACACCTCAG 3'
	5' GAGGAGCTGCAGCAGACAGCT 3'
	5' GCTGGCCGAGCTGGAGGAGGC 3'
antisense	5' GCCACTGCCACTGCCATATCC 3'
	5' CCAGGGCCAGCTTGGTGTTC 3'
	5' TGTCAATCTCGGCTCTCAGCC 3'
	5' TTCTGTCCGGCTGCGGTTGGC 3'
	5' AGTCTTCCACCAGGTCCTGCA 3'
	5' TTCTGCTGCTCCAGGAACCGC 3'

Keratin 14:

sense	5' GGGGGAGCCTATGGGTTGGGG 3'
	5' CTGGACAAGGTGCGTGCTCTG 3'
	5' AGCCTGAAGGAGGAGCTGGCC 3'
	5' GAGCTGAACCGCGAGGTGGCC 3'
	5' GAGCTGCAGTCCCAGCTCAGC
antisense	5' GGTGCGAAGGACCTGCTCGTG 3'
	5' CAGCTGCATGCAGTAGCGACC 3'
	5' ACCATTCCTCGGCATCCTTGC 3'
	5' GAAGTCATCCGCGGCCAGACG 3'
	5' GATCTTCACTTCCAGGTCGGC 3'

Exclusion of Polymorphisms

Family A

Screen for the Mutation in the L12 Linker of Keratin 5 (Arg331-Cys)

PCR reactions (15 µl total volume) were set up in the presence of $\alpha^{32}\text{P}$ ATP.

sense primer (50 µg/ml)	1.5 µl
antisense primer (50 µg/ml)	1.5 µl
genomic DNA	0.5 µl (approx. 0.5 µg)
PCR buffer x 20 (20 mM MgCl ₂)	0.75 µl
DMSO	1.5 µl
20 mM dNTP	0.15 µl
$\alpha^{32}\text{P}$ ATP	0.05 µl (0.5 µ Ci)
DEPC water	9.1 µl
Amplitaq polymerase	0.045 µl (0.009 units)

The PCR conditions were 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 1 min at 60°C , 2 min at 72°C and 1 cycle of 5 min at 72°C.

sense primer: 5' GTCCTCTCCATGGACAACAGC 3'

antisense primer: 5' TGTCAATCTCGGCTCTCAGCC 3'

Family B

Screen for Mutation in the L12 Linker of Keratin 14 (Val270-Met)

25 µl PCR reactions:

sense primer (50 µg/ml)	2.5 µl
antisense primer (50 µg/ml)	2.5 µl
genomic DNA	0.5 µl (approx. 0.5 µg)
PCR buffer x 20 (20 mM MgCl ₂)	1.25 µl
DMSO	2.5 µl
20 mM dNTP	0.25 µl
DEPC water	15.43 µl
Amplitaq polymerase	0.075 µl (0.015 units)

The cycling parameters for the reaction were 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 45 sec at 65°C and 1 min at 72°C and 1 cycle of 5 min at 72°C.

sense primer (wild type): 5' CAGGTGGGTGGAGATGTCAATG 3'

sense primer (mutant): 5' CAGGTGGGTGGAGATGTCAATA 3'

antisense primer: 5' ACCATTCCTCGGCATCCTTGC 3'

Family C

Screen for the Mutation in the 1A Domain of Keratin 5 (Asn193-Lys)

50 µl PCR reactions:

sense primer (50 µg/ml)	5 µl
antisense primer (50 µglml)	5 µl
genomic DNA	1 µl (approx. 1 µg)
PCR buffer x 20 (20 mM MgCl ₂)	2.5 µl
DMSO	5 µl
20 mM dNTP	0.5 µl
DEPC water	30.85 µl
Amplitaq polymerase	0.15 µl (0.03 units)

The cycling parameters for the reaction were 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 45 sec at 60°C and 1 min at 72°C followed by 5 min at 72°C.

sense primer: 5' AAGAGGTGGGAGGCACCTTAG 3' (intron I)

antisense primer: 5' AGTCTTCCACCAGGTCCTGCA 3'

Family D

Screen for Mutation in the 2B Domain of Keratin 14 (Arg388-Cys)

To create a new *Pst*I site in conjunction with the mutation.

50 µl PCR reactions:

sense primer (50 µg/ml)	5 µl
antisense primer (50 µglml)	5 µl
genomic DNA	1 µl (approx. 1 µg)
PCR buffer x 20 (20 mM MgCl ₂)	2.5 µl
DMSO	5 µl
20 mM dNTP	0.5 µl
DEPC water	30.85 µl
Amplitaq polymerase	0.15 µl (0.03 units)

The cycling parameters for the reaction were 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 1 min at 60°C and 1 min at 72°C followed by 5 min at 72°C.

sense primer: 5' GAGCTGAACCGCGAGGTGGCC 3'

antisense primer: 5' AGTGCTTGGGCAGGAGAGGGG 3'

The DNA was purified by isopropanol precipitation (see Chapter 3, 3.9) and re-amplified.

50 µl PCR reactions:

sense primer (50 µg/ml)	5 µl
antisense primer (50 µg/ml)	5 µl
genomic DNA (purified)	0.5 µl
PCR buffer x 20 (20 mM MgCl ₂)	2.5 µl
DMSO	5 µl
20 mM dNTP	0.5 µl
DEPC water	31.35 µl
Amplitaq polymerase	0.15 µl (0.03 units)

The cycling parameters for the reaction were 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 45 sec at 64°C and 1 min at 72°C followed by 5 min at 72°C.

sense primer: 5' GAGCTGCAGTCCCAGCTCAGC 3'

antisense primer: 5' ACTCCTGGTTCTGCTGCTCCATCTCGCTGC 3'

Genetic Linkage Analysis

*Ava*II Restriction Fragment Length Polymorphism (RFLP) of Human Keratin 10

25 µl PCR reactions:

sense primer (50 µg/ml)	2.5 µl
antisense primer (50 µg/ml)	2.5 µl
genomic DNA	0.5 µl (approx. 0.5 µg)
PARR buffer x 10 (Cambio)	2.5 µl
20 mM dNTP	0.25 µl
DEPC water	16.75 µl
Taq Polymerase (Cambio)	0.1 µl (0.5 U)

The PCR conditions were 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C and 1 cycle of 5 min at 72°C.

sense primer: 5' CCTAACAACTGATAATGCCAA 3'

antisense primer 5' AGTTGAGTCAGATCAACACCC 3'

D17S800

Primers were from the Genethon probe bank

CA GGTCTCATCCATCAGGTTTT

GT ATAGACTGTGTACTGGGCATTGA

Primer GT (1µl) was end labelled with $\gamma^{32}\text{P}$ ATP (as for DNA sequencing) and added to the PCR reaction mixture.

primer (7 ng/µl)	1 µl
kinase buffer (x 5 conc.)	1 µl
DEPC water	1 µl
$\gamma^{32}\text{P}$ ATP	1 µl
T4 polynucleotide kinase	1 µl

The primer was incubated for 30 min at 37°C followed by 5 min at 55 °C. This amount of end labelled primer was sufficient for approximately 10 x 25 µl PCR reactions.

PCR reactions were set up as follows:

CA primer (50 µg/ml)	2.5 µl
GT primer (50 µg/ml)	2.5 µl
GT end labelled primer	0.5 µl
genomic DNA	0.5 µl (approx. 0.5 µg)
PCR buffer x 20 (20 mM MgCl ₂)	1.25 µl
20 mM dNTP	0.25 µl
DEPC water	17.5 µl
Amplitaq polymerase	0.075 µl (0.42 units)

The PCR cycling parameters were 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C.

***Bsa*II and *Mae*II Restriction Length Polymorphism (RFLP) of Human Keratin 8**

100 µl PCR reactions:

sense primer (50 µg/ml)	10 µl
antisense primer (50 µg/ml)	10 µl
genomic DNA	1 µl (approx. 1 µl)
PCR buffer x 20 (20 mM MgCl ₂)	5 µl
20 mM dNTP	1 µl
DMSO	10 µl
DEPC water	63 µl
Amplitaq polymerase	0.3 µl (1.67 units)

The PCR conditions were 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C and a final cycle of 5 min at 72°C.

primers: 5' GATATGAGAAGGCTGGTCCTT 3'

5' TTGTTGATAGTACCCACCCCC 3'

Appendix III

Protein Analysis

Materials

Acrylamide and bisacrylamide were obtained from National Diagnostics and the low range (18.5-106 kD) prestained molecular weight markers from Biorad. Coomassie brilliant blue (R-250), BCIP (5-bromo-4-chloro-3-indolylphosphate) and NBT (nitro blue tetrazolium) were purchased from Sigma, the nitrocellulose paper (0.22 μ m) from Schleicher and Schuell. Secondary antibody, rabbit antimouse alkaline phosphatase conjugated antibody (D314) was from DAKO. All other chemicals were purchased from BDH. The gel electrophoresis and Western blotting apparatus were purchased from Hoeffer Scientific Instruments.

Buffers for cytokeratin extraction, SDS polyacrylamide gel electrophoresis and Western blotting

Low salt extraction buffer : *10 mM Tris, 150 mM NaCl, 3 mM EDTA, 0.1% N-P40 pH 7.4.

High salt extraction buffer : * 10 mM Tris, 50 mM NaCl, 1.5 M KCl, 3 mM EDTA , 0.1% N-P40 pH 7.4 .

Wash buffer : * 10 mM Tris, 150 mM NaCl, 3 mM EDTA pH 7.4.

* The following protease inhibitors were added just before use to the extraction buffers and to the wash buffer to reduce keratin degradation.

0.2 mM phenylmethanesulphonyl fluoride PMSF (0.1 M stock dissolved in isopropanol and diluted 1:500). It is stable at room temperature for several months but once diluted PMSF has a half life of only approximately 30 min.

0.5 μ g/ml leupeptin (1 mg/ml stock dissolved in distilled water diluted 1:2000).

Frozen in small aliquots, it can be stored at 4°C for a month.

0.5 µg/ml pepstatin (1 mg/ml stock dissolved in distilled water and diluted 1:2000).

Pepstatin is stable at room temperature for several months.

SDS Sample buffer

2% w/v SDS	0.2 g
10% w/v glycerol	1 g
100 mM dithiothreitol	0.15 g
0.5 M Tris-HCl pH 6.8	1.25 ml
0.02% w/v bromophenol blue	2.0 mg

Made up to 10 ml in distilled water and stored at -20°C in small aliquots.

Resolving gel: Total gel concentration = 10%, Crosslinking = 2%

30 ml

30% w/v acrylamide	9.81 ml
2% w/v bisacrylamide	3.0 ml
1.5 M Tris-HCl pH 8.8	7.5 ml
10% w/v SDS	0.3 ml
distilled water	9.15 ml
TEMED	0.15 ml
10% ammonium persulphate	0.225 ml

Stacking gel: Total gel concentration = 5%, Crosslinking = 2%

5 ml

30% acrylamide	0.82 ml
2% bisacrylamide	0.25 ml
0.5 M Tris-HCl pH 6.8	1.25 ml
10% SDS	0.05 ml
Distilled water	2.58 ml
TEMED	0.005 ml
10% ammonium persulphate	0.0375 ml

SDS PAGE Electrode buffer x 10 concentration: 0.25 M Tris-HCl, 1.92 M glycine, 1% SDS. Diluted 1:10 in distilled water for use.

Western blot transfer buffer

Tris-base	30 g
glycine	144 g
methanol	1.25 l

The Tris-base and glycine were dissolved in distilled water, made up to 3.75 l and the methanol added.

Coomassie stain : 0.25% (w/v) Coomassie brilliant blue (R-250) was dissolved in 50% (v/v) methanol, 10% acetic acid (v/v) and made up to volume with distilled water.

Destain solution : 5% (v/v) methanol, 7.5% (v/v) acetic acid was made up in distilled water.

Primary antibodies for staining the Western blots: the following antibodies were diluted appropriately in tissue culture medium containing 10% FCS.

AE14 to K5	1:10	(Moll <i>et al.</i> , 1988)
LL001 to K14	1:20	(Purkis <i>et al.</i> , 1990)
AE1 to type I keratins	1:50	(Woodcock-Mitchell <i>et al.</i> , 1982)
AE3 to type II keratins	1:20	(Woodcock-Mitchell <i>et al.</i> , 1982)
α IFA to most IFs	1:30	(Pruss <i>et al.</i> , 1981)
PCK26 to K1,5,6,8	1:100	
RCK102 to K5,8	1:5	(Ramaekers <i>et al.</i> , 1987)
E3 to K17	1:5	(Guelstein <i>et al.</i> , 1988)
ME101 to most IFs	1:10	(Ecurat <i>et al.</i> , 1989)
LE41 to K8	1:5	(Lane <i>et al.</i> , 1982)
LP34 to K4,5,6,10,(13,14),18		(Lane <i>et al.</i> , 1985)
LP1K to K1,2,4,5,6,8	1:5	(Lane <i>et al.</i> , 1985)
LE61 to K18	1:5	(Lane, 1982)

Secondary antibody : rabbit anti-mouse alkaline phosphatase conjugated antibody was diluted 1:500 in DMEM medium containing 10% FCS for use.

Wash buffer : 10 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.5% TWEEN-20.

Substrate solution : 0.1 M Tris-HCl pH 9.5, 0.1M NaCl, 5mM MgCl₂ containing 330 μ l NBT (10 mg/ml in water) and 33 μ l BCIP (50 mg/ml in 100% dimethyl formamide) per 10 ml.

Ponceau S : A stock solution of 2% Ponceau S was made up in 30% w/v trichloroacetic acid, and diluted 1:10 in water for use.

India ink : 0.01% India Ink was dissolved in 0.3% TWEEN-20 in PBS.

Appendix IV

UK DEBRA Epidermolysis Bullosa National Register and Blister Diaries

The aim of the UK National EB Register is to collect information from EB patients throughout the country that will provide an invaluable database for medical staff, EB patients and scientists involved in EB research. Data is collected in the form of a questionnaire, together with a clinical examination. From this, answers to many of the unknown questions will be established such as prevalence, frequency of the different subtypes, physical independence, economic and social problems as a direct result of the disease and geographic distribution. Patients in Scotland have been interviewed and clinically examined by either Dr. H. M. Horn or Dr. M. J. Tidman. This section of the UK register is nearing completion. As well as providing a database of accurate and detailed clinical information, families willing to participate in research projects have been identified. In Scotland EB sufferers were traced by contacting Scottish dermatologists and from family contacts. Twenty eight per-cent of EB patients identified had never been seen by a dermatologist. For some of the families this contact has been beneficial in introducing them to DEBRA and the services provided by the organisation such as counselling, advice from the nurse, meetings and social activities.

Preliminary data from the 230 EB patients identified in Scotland shows EB simplex to be the most common form (67%) then dystrophic EB (37%), with only a very few cases of junctional EB (1%). Of the EB simplex patients, 97% have the Weber-Cockayne variant and 3% the more severe Dowling-Meara. Blistering at additional sites to the palms and soles occurred in 59% of the EB simplex patients; 18% had mouth blisters and 14% nail dystrophy. In 6% of the EB simplex patients there was no previous family history of blistering (Horn *et al*, 1994).

For my project, in addition to the information collected for the register, several EBS patients were asked to record the number of blisters that developed over several months to see what factors induced blisters and the range in number of blisters per person per month. A simple questionnaire was designed to record the site, number and frequency of blisters, together with any contributing factors such as the weather or, activities performed. Diaries were returned from nine EBS-WC patients, some from families examined in this study, and had been kept for between 2-7 months. Blisters occurred predominantly on the soles, induced mainly by walking, dancing and various sports activities, and less frequently on the palms and at other body sites. In general blisters were more common in the warmer weather. In some, particularly the adults, very few blisters developed over several months; whether this reflects the suggestion of improvement in the disease with age or merely avoidance of activities known to induce blisters, and the cool climate in Scotland is unknown. However, the children reported more frequent blistering, both in the summer and in the winter. Many of these were obviously induced by physical activities performed throughout the year such as football. It is unknown how long the blisters took to heal and whether some of them reformed, but in general they were not severe enough to result in absence from school or work. Although these results only represent a small sample group and did not reveal any unexpected findings they did confirm the wide variation in the frequency and number of blisters between patients.

Appendix V

Publication: Rugg, E.L., Morley, S.M., Smith, F.J.D., Boxer, M., Tidman, M.J., Navsaria, H., Leigh, I.M. and Lane, E.B. Missing links: Weber-Cockayne keratin mutations implicate the L12 linker domain in effective cytoskeleton function. *Nature Genetics* 5:294-300, 1993a.

Missing links: Weber-Cockayne keratin mutations implicate the L12 linker domain in effective cytoskeleton function

E.L. Rugg¹, S.M. Morley¹, F.J.D. Smith^{1,2}, M. Boxer³, M.J. Tidman², H. Navsaria⁴, I.M. Leigh⁴ & E.B. Lane¹

We have identified mutations in keratins K5 (Arg331Cys) and K14 (Val270Met) in two kinships affected by the dominantly-inherited skin blistering disease, Weber-Cockayne epidermolysis bullosa simplex (EBS-WC). Linkage analysis, DNA sequencing and clinical and ultrastructural analysis are combined to provide the first detailed description of classical EBS-WC. Both phenotypes show similar blistering on trauma, indicating that both mutations compromise the structural resilience of the basal keratinocytes by affecting the keratin cytoskeleton. The location of these mutations in the L12 linker, which bisects the α -helical rod region of intermediate filament proteins, identifies another keratin mutation cluster leading to hereditary skin fragility syndromes.

¹CRC Cell Structure Research Group, Department of Anatomy and Physiology, Cancer Research Campaign Laboratories, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, UK
²Department of Dermatology, Edinburgh Royal Infirmary, Edinburgh EH3 9YW, UK
³Medical Genetics Group, Department of Pathology, Ninewells Hospital, Dundee DD1 9SY, UK
⁴Experimental Dermatology Laboratories, Royal London Hospital Medical College, Whitechapel, London E1 6BL, UK

The recent discovery that inherited skin blistering diseases can be caused by mutations in genes encoding keratin intermediate filament proteins has aroused renewed interest in these structural proteins. The opportunity to "see" keratin filaments in action has led to a better understanding of the extent to which intermediate filaments function as the cell's cytoskeleton. Defects in keratin genes have now been characterised in skin fragility syndromes of varying severity, from the Dowling-Meara form of epidermolysis bullosa simplex (EBS-DM^{1,2}) which can cause fatalities in small babies, to the less severe Koebner type of EBS (EBS-K)³⁻⁵, to a form of epidermolytic hyperkeratosis (EH) known as the Brocq type of bullous congenital ichthyosiform erythroderma (BCIE-Brocq)⁶⁻¹⁰. Patients with EBS show stress-induced rupture of basal keratinocytes in the epidermis, and all the EBS cases identified at the gene level show point mutations in the genes for keratins K5 or K14, which are expressed in these basal cells. In contrast, all the BCIE cases characterised genetically have defects in either keratins K1 or K10, which are the keratins expressed in the suprabasal differentiating keratinocyte layers of the epidermis: in BCIE, it is these suprabasal cells that rupture. All the reports in these types of skin fragility syndromes have been of dominant negative mutations. A case of a recessive skin fragility syndrome has also been reported recently with a mild phenotype resembling that of the dominant Weber-Cockayne type of EBS, which was found to be caused by homozygosity for a point mutation in keratin 14 (ref. 11).

The locations of mutations in different keratin sequences are emerging as clusters, particularly at the helix boundary peptides which mark the consensus ends of the rod domain.

This suggests that fidelity of sequence conservation at these sites may be especially important for optimal alignment and assembly pathways of the proteins, leading to maximal filament network resilience. Conversely, this clustering would also suggest that there may be many undetected mutations within the population which have no pathological implications if they are located at non-critical sites, such as the growing number of identified keratin polymorphisms¹²⁻¹⁵. However, various experiments suggest that many other sites are also important contact points for polymerization¹⁶. One would predict the existence of mild skin defects associated with other groups of mutations that lie between these extremes.

Weber-Cockayne EBS (EBS-WC) is the mildest form of the blistering diseases that involve lysis of epidermal basal cells. In contrast to the clustered and generalized blistering seen in Dowling-Meara and Koebner EBS, the skin damage in EBS-WC patients is limited usually to skin thickening, and some blistering, on the hands and feet. Furthermore, keratin filaments in the cells of affected patients show no ultrastructural abnormalities in EBS-WC, nor with the Koebner form^{17,18}. Linkage studies have mapped familial cases of EBS-WC^{3,19} to chromosome 12, where the type II keratin gene cluster is located, making it likely that this disease is also due to keratin defects. This has led to the suggestion that specific palmo-plantar keratins like K9 may be involved²⁰. An alternative simple explanation would be that less detrimental keratin mutations occur in these patients, such that skin fragility is only apparent at body sites of greatest abrasion and pressure (palms and soles). EBS-WC has not yet been characterized fully at the genetic level, probably due to the fact that this condition is much milder and does not generally justify the taking of

skin biopsies for investigations. Very often, it does not even present clinically, and thus its frequency in the population (estimated at 1:50,000) is hard to ascertain.

Here we describe two kinships affected with EBS-WC, caused by dominant negative mutations in keratin 5 and keratin 14 respectively. Both families carry mutations within the L12 linker domain, a non-helical stretch in the centre of the rod domain in all intermediate filaments. This represents a new site for a mutation cluster in EBS, and casts light on the structural importance of this little-understood subdomain, where experimental mutations have not shown any clear-cut deleterious effects. The implications of this new mutational cluster is significant for our understanding of the assembly of intermediate filaments.

Identification of mutations in the L12 subdomain

The genes for keratins 5 and 14 are located in gene clusters on the long arms of chromosomes 12 and 17, respectively, and polymorphisms in these regions have been linked to EBS-WC^{3,19}. Our analysis of DNA from kinship A for polymorphisms in the COL2A1 gene (12q13) and the COL1A1 gene (17q21.3-q22) indicated that the disease cosegregated with COL1A1 markers on chromosome 17 (Fig. 1a), suggesting the possibility of a defect in K14 since basal cells are affected. The disease did not segregate with chromosome 12, so keratin genes on this chromosome were excluded from further investigation. In kinship B, non-linkage with a keratin 10 polymorphic marker²¹ on chromosome 17 made linkage with any type I keratins unlikely (Fig. 1b), so further study was concentrated on

type II keratin 5 in this case.

In kinship A, a G to A mutation at the first position of codon 270 of K14 was discovered (Fig. 2a), which resulted in a change in the predicted amino acid sequence from valine to methionine. This mutation was present in cDNA from patients IV.4, IV.6 and IV.19 but was not found in other cDNA samples (including 5 unrelated EBS-WC affected patients), nor in genomic DNA from an unaffected member of this family (IV.9), but it was seen in genomic DNA from other affected family members (III.9 and IV.8).

A silent T to C change at the third position of codon 12 of the K14 coding sequence was also identified in patient IV.4 (homozygous) and IV.6 (homozygous) (not shown). The same change was also found in 4 unrelated EBS-WC affected patients (2 homozygous, 2 heterozygous). This variant has been reported previously¹, does not result in an amino acid change, and probably represents a common silent polymorphism.

In kinship B, another mutation was found in the L12 linker domain of keratin 5. This was a C to T mutation at the first position of codon 331, which results in an arginine to cysteine change (Fig. 2b). This mutation was found in cDNA (II.5 and III.4) and in genomic DNA from affected family members (II.3, II.5, III.3, III.4 and III.5). It was not present in genomic DNA from an unaffected family member (II.4) nor in four unrelated genomic DNA samples (3 affected by EBS) nor in five further unrelated cDNA samples (all EBS affected).

We examined nine separate EBS-WC families but found mutations in the L12 linker region in only the two

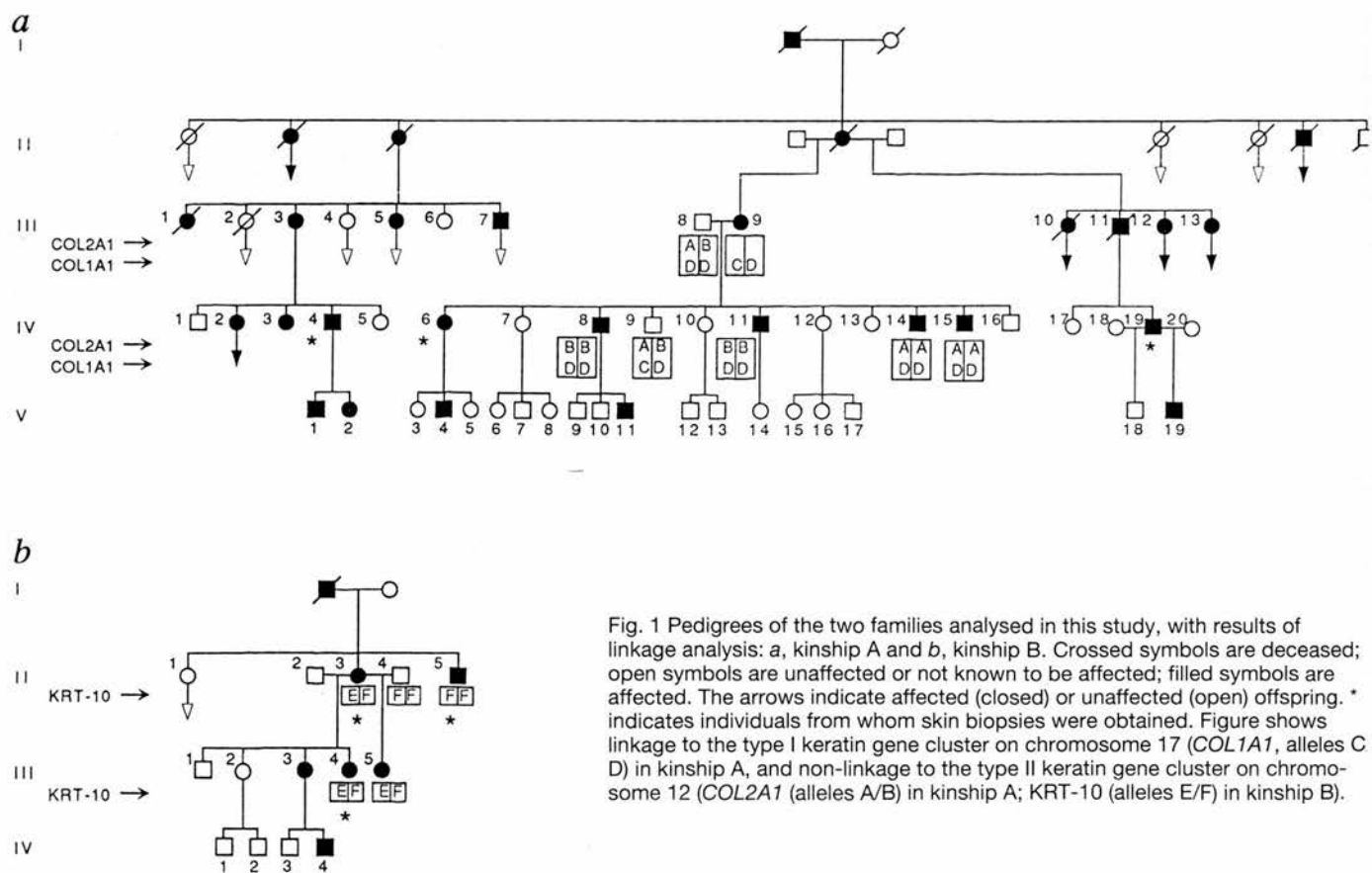


Fig. 1 Pedigrees of the two families analysed in this study, with results of linkage analysis: a, kinship A and b, kinship B. Crossed symbols are deceased; open symbols are unaffected or not known to be affected; filled symbols are affected. The arrows indicate affected (closed) or unaffected (open) offspring. * indicates individuals from whom skin biopsies were obtained. Figure shows linkage to the type I keratin gene cluster on chromosome 17 (COL1A1, alleles C/D) in kinship A, and non-linkage to the type II keratin gene cluster on chromosome 12 (COL2A1, alleles A/B) in kinship A; KRT-10 (alleles E/F) in kinship B).

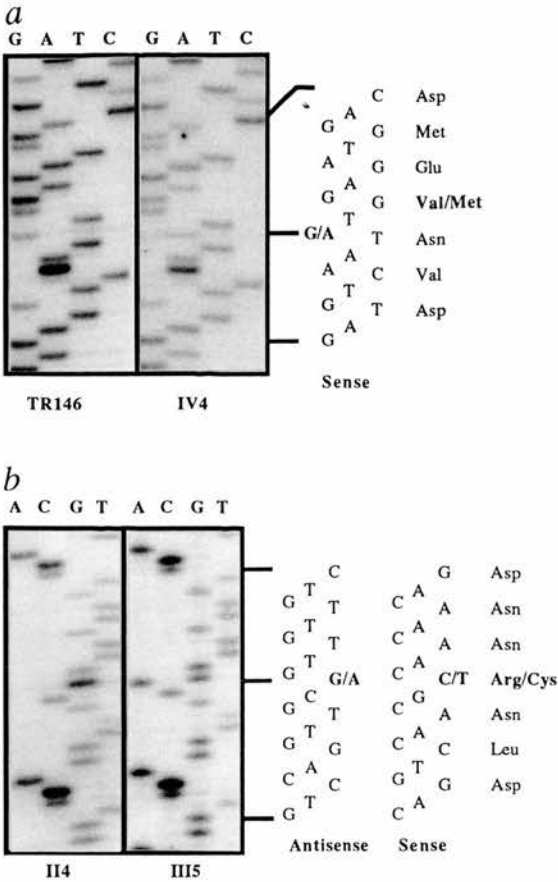
Fig. 2 a, The DNA sequence and predicted amino acid sequence for part of the region encoding the L12 linker of a, keratin14 and b, keratin 5. a, sequence of PCR-amplified keratin 14 cDNA from TR146 (control) and IV.4, kinship A (affected). b, Sequence of PCR-amplified keratin 5 genomic DNA from II.4 (unaffected) and III.5 (affected), kinship B.

described. To exclude the possibility that either of these mutations was an innocuous polymorphism, genomic DNA from unrelated and unaffected controls and from affected and unaffected family members was screened. The screen for the mutation in kinship A was based on the principle that an exact match of the 3' base of the primer is required for the PCR to take place ("PASA")²². By setting the mutant base at the 3' position in the primer, only samples containing the mutant allele are amplified using the primer specific to the mutation. Under the conditions described, all samples were amplified when the normal primer was used, but no product was obtained when the mutant primer was used with samples from unrelated normal controls or unaffected relatives (Fig. 3a). DNA obtained from affected members was amplified, however, with the mutant primer (Fig. 3a). A total of nine affected related individuals, two unaffected related and 54 unaffected unrelated were tested. It is highly unlikely that this mutation is an innocuous polymorphism (χ^2 (1 d.f.)=59.9; $P<0.001$).

A different approach was used to screen controls for the mutation found in kinship B. A primer was designed which, in conjunction with the identified mutation, created a new *AluI* site. After amplification with this primer, genomic DNA from affected family members of kinship B could be cut by *AluI* (Fig. 3b), in contrast to controls of amplified genomic DNA from one unaffected relative (II.4) and from 53 unrelated individuals (5 EBS affected), which were not cut. This makes it highly probable that this Cys331 is the causative mutation of EBS in this family (χ^2 (1 d.f.) = 46.02; $P = <0.001$).

Expression of keratin proteins

No obvious abnormality or deficiency was detected in the pattern of keratin proteins expressed in keratinocytes from these patients. Immunoblotting of cytoskeleton extracts showed the major keratin pairs expressed as



keratins 5 and 14 and keratins 6 and 16 as expected from previous studies²³, together with small but detectable amounts of K1, K10 and K17. There was no detectable reactivity with antibodies to K8 or K18. A normal pattern of reactivity was seen with all antibodies tested: no additional bands were detected, and the migration of keratins extracted from affected patients keratinocytes was indistinguishable from that of unaffected controls (Fig. 4). No variation was seen in the keratin 14 samples from the two families, but in family B, keratin 5 migrated as a doublet in some individuals. This indicates that family B are polymorphic for the keratin 5a/5b alleles (Fig. 4b)^{24,25}, as seen previously in another EBS family². However this polymorphism did not segregate with the disease phenotype.

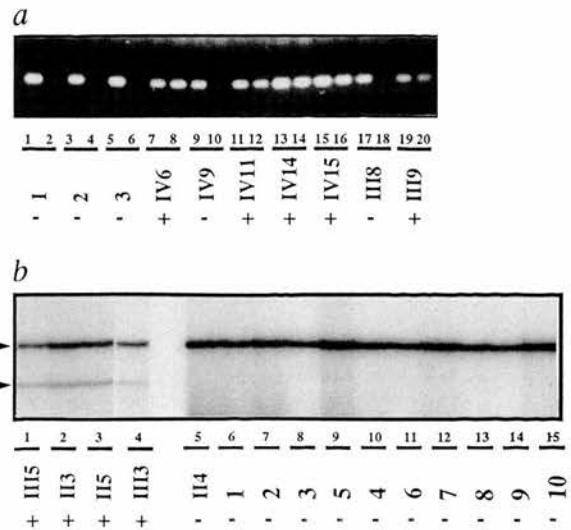


Fig. 3 a, Agarose gel showing PASA analysis of genomic DNA for the G-to-A transition at codon 270 of keratin 14. Lanes 1-6 show the results of amplification of a 130 bp fragment of DNA from 3 unrelated unaffected individuals and lanes 7-20 DNA from 5 affected and 2 unaffected members of kinship A using the wild type primer (odd numbers) and the mutant primer (even numbers). b, Autoradiograph showing the analysis of genomic DNA from 4 affected and 1 unaffected member of kinship B (lanes 1-5) and 10 unrelated unaffected controls (tracks 6-15) for the presence of a new *AluI* site in a PCR-amplified fragment of the keratin 5 gene. The arrows indicate the normal fragment (top) and the fragment produced after digestion with *AluI* (bottom). Samples from EBS affected patients are indicated by +, and unaffected family members or controls by -.

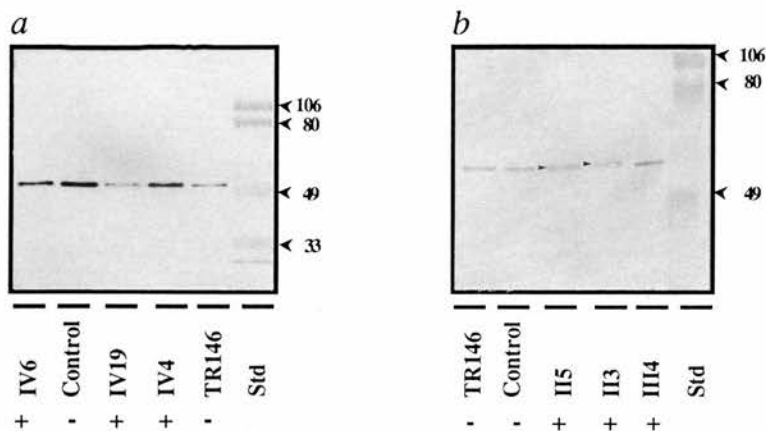


Fig. 4 Immunoblots of cytoskeletal extracts from TR146 cells and primary keratinocyte cultures from an unrelated unaffected control and affected members of *a*, kinship A and *b*, kinship B. *a*, stained with a monoclonal antibody to keratin 14 (LL001) and *b* stained with a monoclonal antibody to keratin 5 (AE14). The arrows (*b*) indicate the K5a polymorphism present in some members of this family. Samples from EBS affected patients are indicated by +, and unaffected controls by -. Prestained molecular weight markers, std, were used to estimate molecular weight; phosphorylase B = 106,000; bovine serum albumin = 80,000; ovalbumin = 49,500; carbonic anhydrase = 32,500 Daltons.

The Weber-Cockayne EBS phenotype

Kinship A is a large Scottish family (>116) which has been affected by hereditary blistering for at least five generations, with a pattern of inheritance consistent with autosomal dominant transmission (Fig. 1*a*). These patients presented as three separate families. Upon closer investigation after identical mutations were found, the families were subsequently deduced to be distantly related. Five affected family members were interviewed using the UK DEBRA Epidermolysis Bullosa Register questionnaire, which records distribution of blisters, age of onset of blistering and environmental factors affecting blistering. The age of onset was usually within one month of birth, but one had blisters at birth (A/IV.19) and another showed no blistering before the age of two years (A/IV.4). All patients reported blisters on their hands and feet (Fig. 5*a,b*) and all except one (A/IV.4) experienced blisters elsewhere, particularly at friction points and when wearing tight clothing. The diagnosis of EBS was confirmed by electron microscopy, which showed intraepidermal blistering with evidence of basal cell cytolysis (Fig. 5*c*). No tonofilament clumping or other keratin filament morphological abnormality was observed in the intact cells.

The second family (kinship B >20), is shown in Fig. 1*b*. Five affected members of this family were interviewed. Although the EBS-WC phenotype is regarded as very mild, it was clear that it nevertheless constituted a significant handicap which caused distress and reduced the quality of life, especially for children. The onset of blistering in kinship B was later than in A, at between 6 and 10 years of age. All patients questioned reported increased blistering during hot weather and a reduced incidence during cold weather.

Discussion

The L12 linker subdomain is assumed to be a highly flexible, non-helical stretch, but its function is so far unknown. The position and number of residues in the L12 subdomain are well conserved in most intermediate

filaments²⁶, and the amino acid sequence shows high homology within each type of intermediate filament (Fig. 6), implying that it is an important structural feature. We have characterized two examples of L12 mutations in EBS-WC, both of which can be seen from the human phenotype to compromise the strength and resilience of the epithelial cytoskeleton. Another mutation in an EBS Koebner family has recently been identified as lying in the region of K14 (ref. 4). The emergence of a mutation cluster in the L12 linker highlights the unexpected sensitivity of this region to structural alterations. The pathological consequences of mutations in the L12 linker however appear to be less profound than for mutations in the helix boundary peptides which result in the most severe forms of EBS^{1,2} and some forms of BCIE⁶⁻⁹.

Attempts to analyse the function of this central linker domain have so far been inconclusive. Gill *et al.* experimentally lengthened the L12 domain in neurofilament, without dramatic effect. McCormick and colleagues produced K14-vimentin hybrids of the rod domains using the linker regions as the splice sites²⁸. Some of these hybrids had an L12 region which was half keratin linker and half vimentin linker. Although hybrids with altered L12 regions resulted in aberrant intermediate filament formation, the contribution of the altered L1 compared to the switched rod domains is not clear. Lett and colleagues systematically mutated all the proline and glycine residues in the L12 linker of keratin 14 so as to make this region completely α -helical²⁹. These substitutions had no observable effect on keratin filament formation when expressed in SCC-13 or PtK2 epithelial cells, but there was a slight tendency towards aggregation during *in vitro* filament formation with wild-type K5. The

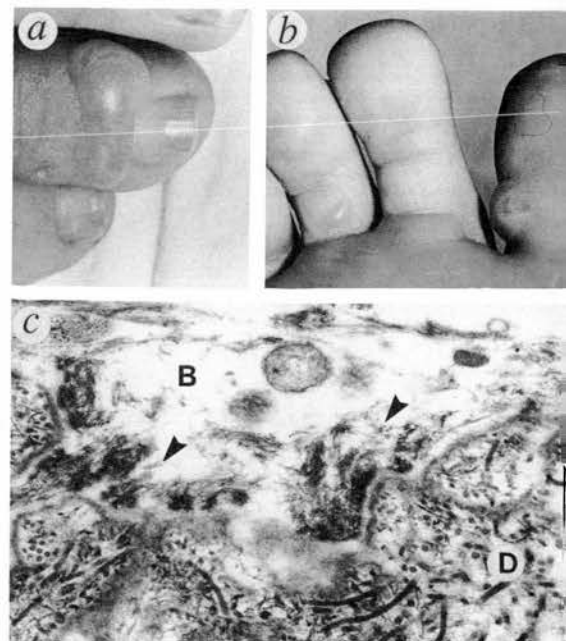


Fig. 5 Examples of blisters on the feet of patient IV.6 (kinship A). Note the presence of blisters on the upper surface of the toe (*a*) as well as on the plantar surface (*b*). *c*, Electron micrograph of the edge of a blister from the same patient. Arrows show remnants of a ruptured basal keratinocyte attached to the basal lamina; B, blister cavity; D, dermis. Magnification x31,600.

lack of dramatic effects in *in vitro* experiments is becoming well recognized now when dealing with intermediate filaments, and reflects the fact that the primary function of these structures is seen in the three-dimensional tissue.

There is also a recent description of a point mutation in a region identified as the L12 linker domain of what is suggested to be a very distantly related intermediate filament protein, which does give a phenotype, the MDM1 protein in the yeast *Saccharomyces cerevisiae*³⁰. A serine to asparagine mutation in codon 257 of this protein is associated with a defect in partitioning of mitochondria and nuclei at mitosis. However it is not clear yet whether this protein can be really classed as an intermediate filament, as it lacks certain key structural features.

Structural implications for keratin filaments. Alternative amino acid sequences for L12 were analysed using GeneWorks version 2.1 (Intelligenetics Inc.), comparing different secondary structure prediction algorithms^{31,32} in order to increase the confidence of α -helicity predictions (Fig. 7). The Val270Met mutation in K14 is predicted to increase the α -helicity in the centre of the L12 linker, which might alter the length or flexibility of this subdomain. In comparison, the experimentally mutated L12 sequence²⁹ is predicted to form a slightly larger region of helicity, and this mutant was demonstrated to affect *in vitro* filament assembly. Interestingly, lamin intermediate filament proteins are thought to have helical linker regions³³, and in *in vitro* polymerisation experiments they do not form filament bundles³⁴.

Similar analysis of the K5 mutation does not predict such an obvious structural change, but the introduction

of a cysteine is potentially a very disruptive change with its size difference and its capacity for cross-linking. This mutation is located closer to the start of the helix 2A domain.

Symmetrical mutation clusters. Figure 6 summarizes the location of the L12 mutations described here in relation to the position of other published keratin mutations in EBS. Multiple pathological mutations associated with skin fragility syndromes of various types have now been described in the rod domain of keratin molecules. These are clustered near the beginning of helix 1A in the "helix initiation peptide" of type I keratins K14 (ref. 1,7) and K10 (refs 7,9), and now in a type II keratin K1 (ref. 10); and also in the helix termination peptide of type II keratins K5 (refs 2,5) and K1 (ref. 9). In addition, mutations have been reported in the H1 region of the head domain in type II keratin 1 (ref. 8) and K5 (ref. 35), and in a recessive mutation in the latter part of helix 1A (type I keratin K14)¹¹, and in helix 2B (K14)³.

This report has added another site to this list, at the centre of the keratin molecule in the linker subdomain L12, which brings the number of candidate mutation regions to six. Excluding the mutation in the H1 domain of the head region which appears to be of a different nature, the addition of the L12 region to the existing group gives an axis of symmetry of the mutation clusters within the keratin molecule, suggesting that they reflect a regular feature of subunit assembly. Current knowledge of subunit interactions in filament assembly is insufficient to determine what the interactions at these sites are, and further study of these mutated

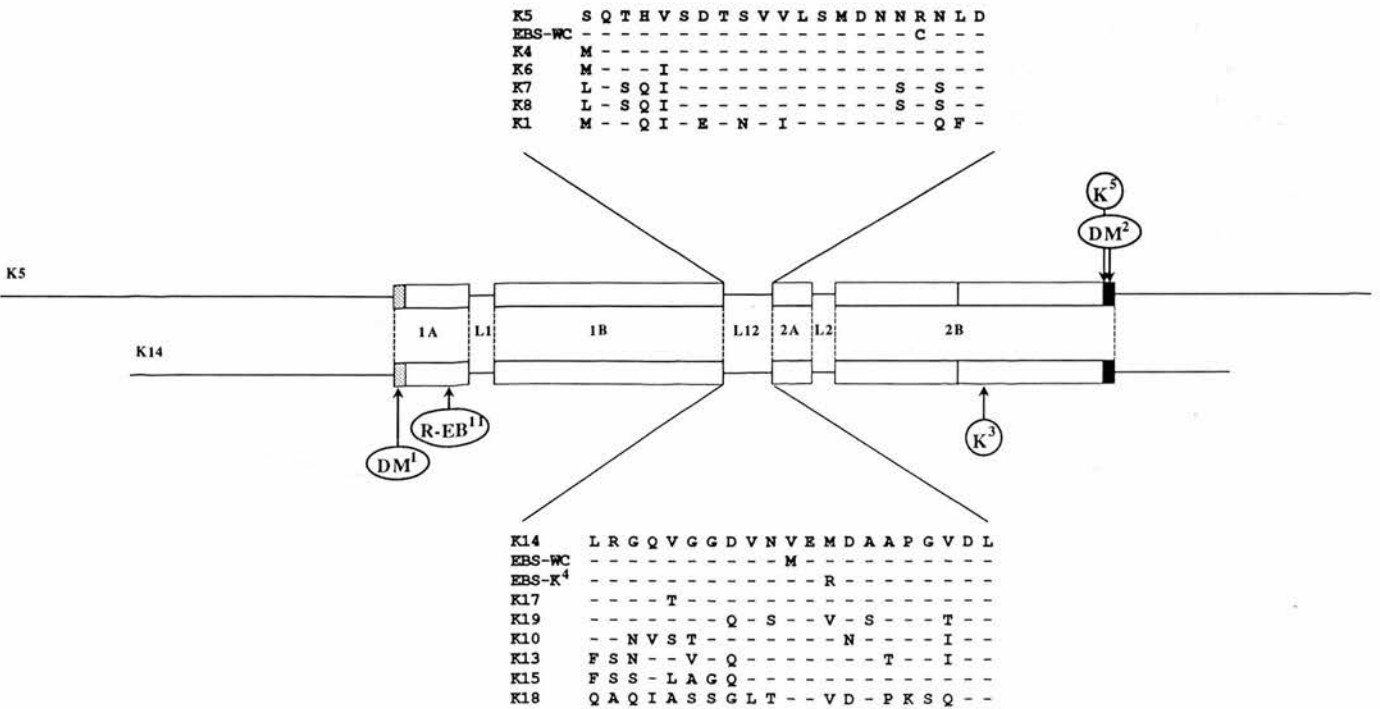


Fig. 6 Diagrammatic representation of keratins 5 and 14 to show the positions EBS-WC mutations, relative to previously reported mutations in patients affected by other variants of EBS (K³⁻⁵, DM^{1,2} and R-EB¹¹). The boxes indicate the four conserved α -helical subdomains (1A, 1B, 2A and 2B) and the shaded areas the conserved helix initiation peptide (N-terminal end of helix) and the consensus helix termination peptide (C-terminal end of helix). The helical subdomains are connected by non-helical linker regions (L1, L12 and L2) and the rod domain is flanked by non-helical N-terminal head and C-terminal tail domains. The vertical line in helix 2B represents a conserved helix phase reversal. The L12 region is expanded to show the amino acid sequence alignment of type I and type II keratins and the mutations in K5 and K14.

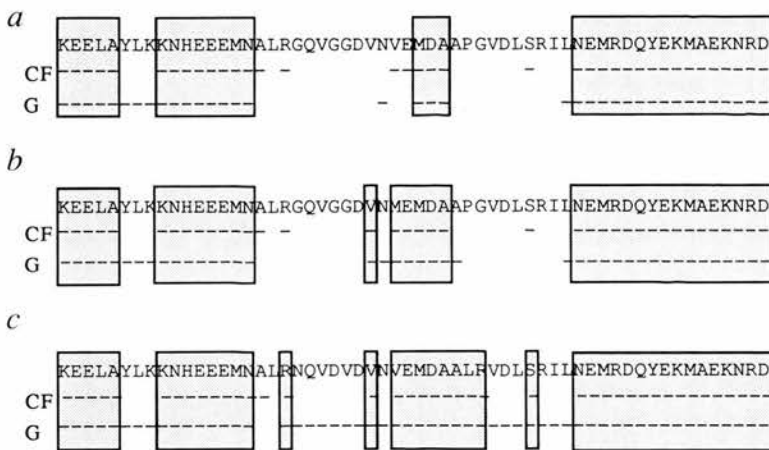


Fig. 7 The L12 amino acid sequence of K14 showing the regions predicted to be α -helical (boxes) by both the Garnier (G) and Chou and Fasman (CF) algorithms. The wild-type K14 sequence (a) is compared to the EBS-WC mutation in K14 described in this paper (b) and with the experimentally mutated K14 L12 sequence constructed by Letai and colleagues²⁹ (c). Both mutations increase the prediction of α -helicity.

proteins will be very valuable.

Point mutations and the dominant-negative phenotype.

The intermediate filament cytoskeleton in the epidermis is formed by the non-covalent interaction of keratin molecules. Keratins are obligate heteropolymers, forming a type I-type II heterodimer at the first stage of filament assembly, and if even a small amount of one of these copolymerizing proteins is defective, this can be sufficient to disrupt the whole filament network³⁶. Thus it is not surprising that the keratin mutations identified so far, with one exception¹¹, show a dominant-negative phenotype. Any gene therapy approaches to these diseases may therefore require knockout of the affected gene rather than supplementation of the phenotype with additional normal keratin genes.

All the keratin mutations characterized so far are missense point mutations. Mutations affecting the early stages of filament assembly would be expected to have the most severe effects; more subtle effects might be expected from mutations in areas involved in higher order assembly processes. Alterations in the overall strength of the network might be expected with little or no effect on the form of the intermediate filaments: no ultrastructural filament abnormalities are characteristically associated with either EBS-WC or EBS-K^{17,18}. The contribution of individual residues to the overall integrity of the system is emphasised by comparing the phenotype of the K14 mutation reported here with that studied by Humphries *et al.*⁴. Although these mutations are within two amino acid residues of each other the Met272Arg mutation results in a more severe phenotype than the Val270Met described here.

It is now realistic to view all the EBS diseases as lying on a continuum of clinical severity which depends on the structural implications of each mutation. Clinically, the distinction between EBS-WC and EBS-K can be difficult for this reason. EBS-WC is the mildest form, then EBS-K, and EBS-DM the most severe³⁷. One of the kinships reported here has a relatively severe phenotype for EBS-WC (blisters in places other than the hands and feet, and most patients blistering in early postnatal life): this family might easily have been diagnosed as being affected by a

mild form of EBS-K. It is interesting to note that the late onset of blistering in one patient (IV.4) was accompanied by a less widespread distribution of blisters (hands and feet only). This raises the possibility of the interaction of the disease with some other, as yet undetermined, factor(s).

The clustering of pathological mutations which is emerging from continued analyses of the keratin-dependant skin fragility syndromes suggests that the sites at which changes are pathological are defined and limited, possibly corresponding to major contact sites between oligomers during the polymerisation of keratins. Conversely, there may be many undiscovered mutations in other regions of keratins whose effects range from subclinical to neutral. For example, it has been clearly established that several of the keratin genes are polymorphic¹³⁻¹⁵, and sequence variation between polymorphic alleles may account for some of the individual variation in skin resilience (for example, susceptibility to friction blisters) observed within the population.

Methodology

Clinical assessment. Four families presenting independently were diagnosed as affected with EBS-WC. At the time of diagnosis and initial studies, the full pedigrees were not known, and three of these patients were erroneously believed to be unrelated (Fig. 1a). The diagnosis of EBS-WC in the two kinships was made initially on the basis of clinical examination. Some family members (A/IV.4, A/IV.19, A/V.1, A/V.2, A/V.19, B/II.3, B/II.5, B/III.3, B/III.4, B/III.5; Fig. 1) were further questioned using the UK DEBRA Register questionnaire. Diagnosis of the simplex form of EB was subsequently confirmed by histopathological and ultrastructural examination.

Linkage studies. DNA was extracted from peripheral blood lymphocyte samples obtained from 5 affected (III.9, IV.8, IV.11, IV.14 and IV.15) and 2 unaffected (III.8 and IV.9) family members of kinship A (Fig. 1a), and 4 affected (II.3, II.5, III.4 and III.5; Fig. 1b) and 1 unaffected (II.4) family members of kinship B (Fig. 1b). Kinship A was analysed for polymorphisms in the *COL1A1* locus on chromosome 17 (ref. 38) and the *COL2A1* locus on chromosome 12 (ref. 39). Kinship B was analysed for *Ava* II polymorphism in keratin 10 (ref. 21).

Keratinocyte culture. Skin biopsies obtained from patients IV.4, IV.6 and IV.19 in kinship A (Fig. 1a) and II.3, II.5 and III.4 in kinship B (Fig. 1b), and an unrelated unaffected control were used to produce primary keratinocytes cultures⁴⁰. Keratins⁴¹ and poly (A)+ mRNA (QuickPrep™ Micro mRNA Purification Kit, Pharmacia) were extracted from these cultures and from the established keratinocyte cell line, TRI46 cells⁴².

Immunoblotting. The cytokeratins were analysed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting⁴. Antibodies tested were PCK26 to K1, 5, 6, 8 (Sigma/Biomakor); AE1 to type I keratins; A45B/B3 to K1/2, 5, 8, 18; E3 to K17; a1FA to most IFs; AE14 to K5; LE41 to K8; LE61 to K18; LH1 and LH2 to K10; LL001 to K14; LL025 to K16; LP1K to K7; LP34 to K5, 6, 18; ME101 to most intermediate filaments; RCK 102 to K5, K8; RCK 105 to K7; RSKE60 to K10. For specificity of reaction, see ref. 43 and refs therein.

mRNA extraction, PCR and DNA sequencing. Poly (A)+ mRNA, extracted from keratinocyte cultures was reverse transcribed using 21-base oligonucleotides (K5: 5'-GCTCTTCCGGG-AGGAGGAGGT-3'; 1495-1515; EMBL accession number M19723; K14: 5'-AGTGCTTGGGCGAGGAGGGG-3', 4788-4808; EMBL accession number J00124), as described². cDNA was amplified by PCR⁴⁴ using the same primers as for the reverse transcription reaction and a second primer (K5: 5'-GGTGGTGGCTTTGGGCTCGGT-3'; 88-108; K14: 5'-GGGGGAGCCTATGGGTTGGGG-3', 496-516). The incubations consisted of 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 1 min at 60 °C, and 2 min at 72 °C. The PCR products were purified from contaminating primers by differential

Acknowledgements

We thank Jane Spencer for her technical assistance and Helen Horne for her help in collecting the patient data. This work was supported by CRC Programme Grant no. SP2060. F.J.D.S. is supported by a research studentship from the Dystrophic Epidermolysis Bullosa Research Association (DEBRA).

precipitation with isopropanol⁴⁵. The purified cDNA was sequenced directly using a dsDNA cycle sequencing protocol (BRL). Cycling parameters for the sequencing reactions were 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C for 20 cycles followed by 30 s at 94 °C and 1 min at 72 °C for 10 cycles. The following primers were used for sequencing. Keratin 5: sense; 5'-GGTGGTGGCTTTGGGCTCGGT-3', 88-108; 5'-GTGGAGCTGGAGGCCAAGGTT-3', 613-633; 5'-CGCATGTCTCTGACACCTCAG-3', 701-721; 5'-GCTGGCCGAGCTGGAGGAGGC-3', 1047-1067; antisense; 5'-GCCAC-TGCCACTGCCATATACC-3', 1253-1275; 5'-CCAGGGCCAGC-TTGGTGTTC-3', 1124-1144; 5'-TGTCATCTCGGC-TCTCAGCC-3', 938-958; 5'-TTCTGTCCGGCTGCGGTGGC-3', 802-822; 5'-TTCTGTCTCCAGGAACCGC-3', 312-332. Keratin 14: sense; 5'-CTGGACAAGGTGGCTGCTCTG-3', 706-726; 5'-ACAGCCACAGTGGACAATGCC-3', 2106-2126; 5'-AGCC-TGAAGGAGGAGCTGGCC-3', 2857-2877; antisense: 5'-GGTGCAGGACCTGCTCGTG-3', 4695-4715; 5'-CAGCT-GCATGCAGTAGCGACC-3', 3737-3757; 5'-ACCATTCCTC-GGCATCCTTGC-3', 3366-3386; 5'-GAAGTCATCCGCGG-CCAGACG-3', 2154-2174; 5'-GATCTTCACTTCCAGTCCGGC-3', 739-759.

Received 29 June; accepted 10 August 1993.

Exclusion of polymorphism. Keratin 14 mutation: from kinship A, DNA from 9 family members, 7 affected (III.9, IV.6, IV.8, IV.11, IV.14, IV.15, and V.4) and 2 unaffected (III.8 and IV.9) and 54 unrelated unaffected controls was analysed using PCR amplification of specific alleles (PASA)²². A 130-bp fragment was amplified by PCR using the antisense primer 5'-ACCATTCCTCGGCATCCTTGC-3', 3366-3386 and either 5'-CAGGTGGGTGGAGATGTCAATG-3', 3257-3278 (wild type) or 5'-CAGGTGGGTGGAGATGTCAATA-3' (mutant). Cycling parameters for the reaction were 30 s at 94 °C, 45 s at 65 °C, and 1 min at 72 °C for 30 cycles; PCR products were resolved by agarose gel electrophoresis. Keratin 5 mutation: within kinship B, DNA from 6 family members, 5 affected (II.3, II.5, III.3, III.4 and III.5) and 1 unaffected (II.4), 5 unrelated EBS patients and 48 unrelated unaffected controls was amplified using the following primers; 5' GTCCTCTCCATGGACAACAGC 3', 724-744 (sense) and 5' TGTCAATCTCGGCTCTCAGCC 3', 938-958 (antisense) in the presence of [α -³²P]-ATP (0.05 μ Ci/15 μ l reaction mix) using the same PCR conditions as described. The samples were incubated with 5 mM spermidine for 10 min at 65 °C, cooled and then incubated with *AluI* (1 U/15 μ l) overnight at 37 °C. After incubating for 5 min at 90 °C, the samples were resolved on a 4% sequencing gel⁴⁵.

1. Coulombe, P.A. *et al.* Point mutations in human keratin 14 genes of epidermolysis bullosa simplex patients: Genetic and functional analysis. *Cell* **66**, 1301-1311 (1991).
2. Lane, E.B. *et al.* A mutation in the conserved helix termination peptide of keratin 5 in hereditary skin blistering. *Nature* **356**, 244-246 (1992).
3. Bonifas, J.M., Rothman, A.L. & Epstein, E.H. Epidermolysis bullosa simplex: evidence in two families for keratin gene abnormalities. *Science* **254**, 1202-1205 (1991).
4. Humphries, M.M. *et al.* A mutation (Met-to-Arg) in the type I keratin (K14) gene responsible for autosomal dominant epidermolysis bullosa simplex. *Hum Mutat* **2**, 37-42 (1993).
5. Dong, W., Ryyanen, M. & Uitto, J. Identification of a leucine-to-proline mutation in the keratin 5 gene in a family with the generalized K6bner type of epidermolysis bullosa simplex. *Hum Mutat* **2**, 94-102 (1993).
6. Bonifas, J.M., Bare, J.W., Chen, M.A., Niemi, K.M. & Epstein, E.H. Epidermolysis hyperkeratosis: linkage to keratin gene regions on chromosomes 12q and 17q in two families. *J. invest. Dermatol.* **98**, 573a (1992).
7. Cheng, J. *et al.* The genetic basis of epidermolysis hyperkeratosis: a disorder of differentiation-specific epidermal keratin genes. *Cell* **70**, 811-819 (1992).
8. Chipcev, C.C. *et al.*, Korge, B.P., Markova, N., Bale, S.J., DiGiovanna, J.J., Compton, J.C. & Steinert, P.M. A leucine-proline mutation in the H1 subdomain of keratin 1 causes epidermolysis hyperkeratosis. *Cell* **70**, 621-628 (1992).
9. Rothnagel, J.A. *et al.* Mutations in the rod domains of keratins 1 and 10 in epidermolysis hyperkeratosis. *Science* **257**, 1128-1130 (1992).
10. McLean, W.H.I., Eady, R.A.J., Leigh, I.M., Morley, S.M. & Lane, E.B. A point mutation in helix 1A of keratin 1 creates a Mae III RFLP and causes BCIE/ EHK. *J. invest. Dermatol.* **100**, 516 (1993).
11. Hovnanian, A. *et al.*, Pollack, E., Hilal, L., Rochat, A., Prost, C., Barrandon, Y. & Goosens, M. A missense mutation in the rod domain of keratin 14 associated with recessive epidermolysis bullosa simplex. *Nature Genet.* **3**, 327-332 (1993).
12. Wild, G.-A. & Mischke, D. Variation and frequency of cytokeratin polypeptide patterns in human squamous non-keratinizing epithelium. *Exp. Cell Res.* **162**, 114-126 (1986).
13. Korge, B.P., Gan, S.-Q., McBride, O.W., Mischke, D. & Steinert, P. Extensive size polymorphism of the human keratin 10 chain resides in the C-terminal V2 subdomain due to variable numbers and sizes of glycine loops. *Proc. natn. Acad. Sci. U.S.A.* **89**, 910-914 (1992).
14. Korge, B.P., Compton, J.G., Steinert, P.M. & Mischke, D. The two size alleles of human keratin 1 are due to a deletion in the glycine-rich carboxyl-terminal V2 subdomain. *J. invest. Dermatol.* **99**, 697-702 (1992).
15. Wanner, R., Förster, H.-H., Tilmans, I. & Mischke, D. Allelic variations of human keratins K4 and K5 provide polymorphic markers within the type II keratin gene cluster on chromosome 12. *J. invest. Dermatol.* (1993).
16. Steinert, P.M., Marekov, L.N., Fraser, R.D.B. & Parry, D.A.D. Keratin intermediate filament structure: crosslinking studies yield quantitative information on molecular dimensions and mechanism of assembly. *J. molec. Biol.* (1993).
17. Anton-Lamprecht, I. & Schnyder, U.W. Epidermolysis bullosa herpetiformis Dowling-Meara: report of a case and pathomorphogenesis. *Dermatol.* **164**, 221-235 (1982).
18. Niemi, K.-M., Kero, M., Kanerva, L. & Mattila, R. Epidermolysis bullosa simplex: a new histologic subgroup. *Arch. Dermatol.* **119**, 138-141 (1983).
19. Hoyheim, B., Gedde-Dahl, T. & Olaisen, B. Linkage studies in epidermolysis bullosa simplex. *J. invest. Dermatol.* **98**, 397a (1992).
20. Epstein, E.H. Molecular genetics of epidermolysis bullosa. *Science* **256**, 799-804 (1992).
21. McLean, W.H.I. & Lane, E.B. A II RFLP of human keratin 10 (KRT-10) detected by PCR. *Hum. molec. Genet.* **1**, 659 (1992).
22. Sommer, S.S., Groszbach, A.R. & Bottema, C.D.K. PASA is a general method for rapid detection of known single-base changes. *Biotechniques* **12**, 82-87 (1992).
23. Weiss, R.A., Eichner, R. & Sun, T.-T. Monoclonal antibody analysis of keratin expression in epidermal diseases: a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes. *J. cell Biol.* **98**, 1397-1406 (1984).
24. Mischke, D. & Wild, G. Polymorphic keratins in human epidermis. *J. invest. Dermatol.* **88**, 191-197 (1987).
25. Mischke, D., Wille, G. & Wild, A.G. Allele frequency and segregation of human polymorphic keratins 4 and 5. *Am. J. hum Genet.* **46**, 548-552 (1990).
26. Steinert, P.M. & Parry, D.A.D. Intermediate filaments: conformity and diversity of expression and structure. *Ann. Rev. cell Biol.* **1**, 41-65 (1985).
27. Gill, S.R., Wong, P.C., Monteiro, M.J. & Cleveland, D.W. Assembly properties of dominant and recessive mutations in the small mouse neurofilament (NF-L) subunit. *J. cell Biol.* **111**, (1990).
28. McCormick, M.B., Coulombe, P.A. & Fuchs, E. Sorting out IF networks: consequences of domain swapping on IF recognition and assembly. *J. cell Biol.* **113**, 1111-1124 (1991).
29. Letai, A., Coulombe, P.A. & Fuchs, E. Do the ends justify the means? Proline mutations at the ends of the keratin coiled-coil rod segment are more disruptive than internal mutations. *J. cell Biol.* **116**, 1181-1195 (1992).
30. McConnell, S.J. & Yaffe, M.P. Intermediate filament formation by a yeast protein essential for organelle inheritance. *Science* **260**, 687-689 (1993).
31. Garnier, J., Osguthorpe, D.J. & Robson, B. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. molec. Biol.* **120**, 97-120 (1978).
32. Chou, P.Y. & Fasman, G.D. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* **47**, 45-148 (1978).
33. Conway, J.F. & Parry, D.A.D. Intermediate filament structure: 3. Analysis of sequence homologies. *Int. J. biol. Macromol.* **10**, 79-98 (1988).
34. Heitlinger, E., Peter, M., Häner, M., Lustig, A., Aebi, U. & Nigg, E.A. Expression of chicken lamin B2 in E. coli: Characterization of its structure, assembly and molecular interactions. *J. Cell Biol.* **113**, 485-495 (1991).
35. Rugg, E. *et al.* Skin fragility in Weber-Cockayne EBS caused by mutations in epidermal keratins. *Brit. J. Dermatol.* (in the press).
36. Coulombe, P.A., Chan, Y.M., Albers, K. & Fuchs, E. Deletions in epidermal keratins leading to alterations in filament organization in vivo and in intermediate filament assembly in vitro. *J. Cell Biol.* (1990).
37. Gedde-Dahl Jr., T. Epidermolysis bullosa simplex (intraepidermal epidermolysis bullosa) and allied conditions. *Manag. blistering Diseases 1* (eds Wojnarowska, F. & Briggaman, R.A.) 189-211 (Chapman and Hall Medical, London, 1990).
38. Baker, R. *et al.* PCR detection of five restriction site dimorphisms at the type I collagen loci of COL1A1 and COL1A2. *Nucl. Acids Res.* **19**, 4315 (1991).
39. Priestley, L., Kumar, D. & Sykes, B. Amplification of the COL2A1 3' variable region used for segregation analysis in a family with the Stickler syndrome. *Hum. Genet.* **85**, 525-526 (1990).
40. Rheinwald, J.G. & Green, H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6**, 331-344 (1975).
41. Stasiak, P.C., Purkis, P.E., Leigh, I.M. & Lane, E.B. Keratin 19: Predicted amino acid sequence and broad tissue distribution suggest it evolved from keratinocyte keratins. *J. invest. Dermatol.* **92**, 707-716 (1989).
42. Rupniak, H.T. *et al.* Characteristics of four new human cell lines derived from squamous cell carcinomas of the head and neck. *J. natn. Cancer Inst.* **75**, 621-635 (1985).
43. Lane, E.B. & Alexander, C.M. Use of keratin antibodies in tumor diagnosis. *Sem. Cancer Biol.* **1**, 165-179 (1990).
44. Bartek, J. *et al.* Patterns of expression of the p53 tumour suppressor in human breast tissues and tumours in situ and in vitro. *Int. J. Cancer* **46**, 839-844 (1990).
45. Sambrook, J., Fritsch, E.F. & Maniatis, T. *Molecular Cloning - A laboratory manual* (Cold Spring Harbor Laboratory, New York, 1989).